

**FLUORESCENT QUENCHING DETECTION REAGENTS AND
METHODS**

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to oligonucleotide-quencher-fluorescent-dye conjugates having improved characteristics, and to reagents suitable for incorporating novel quencher and fluorescent dye moieties into oligonucleotides. The invention also relates to the use of oligonucleotide-quencher-fluorescent-dye conjugates in detection methods for nucleic acid targets.

2. Brief Description of Related Art

Synthetic oligonucleotides have been used for years as sequence specific probes for complementary DNA and RNA targets. These methods have broad application in forensics, molecular biology and medical diagnostics since they allow the identification and quantitation of specific nucleic acid targets. Early uses of DNA probes relied on radioactivity (typically ^{32}P) as the label, while recent methods use reporter molecules which include chemiluminescent and fluorescent groups. Improved instrumentation has allowed the sensitivity of these spectroscopic methods to approach or surpass the radiolabeled methods. Recently developed detection methods employ the process of fluorescence resonance energy transfer (FRET) for the detection of probe hybridization rather than direct detection of fluorescence intensity. In this type of assay, FRET occurs between a donor fluorophore (reporter) and an acceptor molecule (quencher) when the absorption spectrum of the quencher molecule overlaps with the emission spectrum of the donor fluorophore and the two molecules are in close proximity. The excited-state energy of the donor fluorophore is transferred to the neighboring acceptor by a resonance dipole-induced dipole interaction,

1 which results in quenching of the donor fluorescence. If the acceptor
2 molecule is a fluorophore, its fluorescence may sometimes be increased. The
3 efficiency of the energy transfer between the donor and acceptor molecules is
4 highly dependent on distance between the molecules. Equations describing
5 this relationship are known. The Forster distance (R_0) is described as the
6 distance between the donor and acceptor molecules where the energy transfer
7 is 50% efficient. Other mechanisms of fluorescence quenching are also
8 known, such as, collisional and charge transfer quenching.

9 Typically detection methods based on FRET are designed in such a
10 way that the donor fluorophore and acceptor molecules are in close proximity
11 so that quenching of the donor fluorescence is efficient. During the assay, the
12 donor and acceptor molecules are separated such that fluorescence occurs.
13 FRET-based detection assays have been developed in the fields of nucleic
14 acid hybridization and enzymology. Several forms of the FRET hybridization
15 assays are reviewed (Nonisotopic DNA Probe Techniques, Academic Press,
16 Inc., San Diego 1992, pp. 311-352).

17 Since its discovery, the polymerase chain reaction (PCR) has
18 revolutionized molecular biology. This technique allows amplification of
19 specific DNA sequences, thus allowing DNA probe assays to be executed
20 from a single DNA target copy. PCR-based diagnostic assays have initially
21 not been used routinely, in part due to problems with sample handling and
22 possible contamination with non-source DNA. Recently, new homogeneous
23 fluorescent-based DNA assays have been described which can detect the
24 progress of PCR as it occurs ("real-time" PCR detection) using
25 spectrofluorometric temperature cyclers. Two popular assay formats use
26 DNA probes which become fluorescent as DNA amplification occurs
27 (fluorogenic probes).

28 The first format for "real-time" PCR uses DNA probes known as
29 "molecular beacons" (Tyagi *et al.*, Nat. Biotech., 16: 49-53 (1998)).
30 Molecular beacons have a hairpin structure wherein the quencher dye and

1 reporter dye are in intimate contact with each other at the end of the stem of
2 the hairpin. Upon hybridization with a complementary sequence, the loop of
3 the hairpin structure becomes double stranded and forces the quencher and
4 reporter dye apart, thus generating a fluorescent signal. *Tyagi et al.* reported
5 use of the non-fluorescent quencher dyes including the dabcyl (4-[[4-
6 (dimethylamino)phenyl]diazenyl}benzoyl moiety, absorbance max=453 nm)
7 used in combination with fluorescent reporter dyes of widely varying
8 emission wavelength (475-615 nm). At the time this was surprising since
9 FRET requires significant overlap of the absorption spectrum of the quencher
10 and of the emission spectrum of the reporter. In case of a dabcyl moiety
11 containing (hereinafter “dabcyl”) quencher and some fluorescent dyes, the
12 spectral overlap was extremely low, yet quenching efficiency was high.
13 Therefore it was proposed that the mechanism of quenching for the hairpin
14 form of the beacons was not FRET, but collisional quenching. In fact, the UV
15 spectra of the quencher changes in the hairpin form of the beacon, providing
16 evidence of the molecular contact and thus of collisional quenching. A
17 related detection method uses hairpin primers as the fluorogenic probe
18 (Nazarenko et al., *Nucl. Acid Res.*, 25: 2516-2521 (1997)).

19 The second format for “real-time” PCR uses DNA probes which are
20 referred to as “5'-nuclease probes” (*Lee et al.*, *Nucl. Acid Res.*, 21: 3761-
21 3766 (1993)). These fluorogenic probes are typically prepared with the
22 quencher at the 3' terminus of a single DNA strand and the fluorophore at the
23 5' terminus. During each PCR cycle, the 5'-nuclease activity of *Taq* DNA
24 polymerase cleaves the DNA strand, thereby separating the fluorophore from
25 the quencher and releasing the fluorescent signal. The 5'-nuclease assay
26 requires that the probe be hybridized to the template strand during the primer
27 extension step (60-65°C). They also disclose the simultaneous “real-time”
28 detection of more than one polynucleotide sequence in the same assay, using
29 more than one fluorophore/quencher pair. The 5'-nuclease PCR assay is
30 depicted in **Figure 1**.

1 Initially it was believed that 5'-nuclease probes had to be prepared with
2 the quencher (usually tetramethylrhodamine (TAMRA)) positioned at an
3 internal nucleotide in close proximity to the 5'-fluorophore (usually
4 fluorescein (FAM) or tetrachlorofluorescein (TET)) to get efficient FRET.
5 Later it was found that this is not necessary, and the quencher and the
6 fluorophore can be located at the 3' and 5' end of the ODN, respectively. It
7 has been proposed that the random coil structures formed by these fluorogenic
8 probes in solution allow a 3'-quencher dye to pass within the Forster radius of
9 the 5'-fluorophore during the excited state of the molecule.

10 A number of donor/acceptor pairs have previously been described,
11 important to the present invention is dabcyI that is used for instance as a
12 quencher of dansyl sulphonamide in chemosensors (*Rothman & Still* (1999)
13 *Med. Chem. Lett.* 22, 509 - 512).

14 Surprisingly, there have been no published reports on the use of dabcyI
15 in 5'-nuclease probes or other FRET probes that use long wavelength
16 fluorophores. As mentioned above, dabcyI was used in the beacon-type
17 probes but this is a different quenching mechanism wherein the dabcyI and
18 fluorophore are in intimate contact (collisional quenching). DabcyI was used
19 in fluorogenic peptides as a quencher for the fluorophore EDANS (5-[(2-
20 aminoethyl)amino]naphthalene-1-sulfonic acid) which emits at short (490 nm
21 , blue) wavelength (*Matayoshi et al. Science* 247: 954-958 (1990)). EDANS
22 also has a lower extinction coefficient than dabcyI so it is not surprising that
23 fluorescent quenching was efficient. It was found for the first time in the
24 present invention that dabcyI can be used to quench fluorescein in a FRET
25 type mechanism.

26 In addition to the 5'-nuclease PCR assay, other formats have been
27 developed that use the FRET mechanism. For example, single-stranded
28 signal primers have been modified by linkage to two dyes to form a
29 donor/acceptor dye pair in such a way that fluorescence of the first dye is
30 quenched by the second dye. This signal primer contains a restriction site

1 (United States Patent No. 5,846,726) that allows the appropriate restriction
2 enzyme to nick the primer when hybridized to a target. This cleavage
3 separates the two dyes and a change in fluorescence is observed due to a
4 decrease in quenching. Non- nucleotide linking reagents to couple
5 oligonucleotides to ligands have also been described (United States Patent
6 No. 5,696,251).

7 FRET systems also have applications in enzymology. Protease
8 cleavable substrates have been developed where donor/acceptor dye pairs are
9 designed into the substrate. Enzymatic cleavage of the substrate separates the
10 donor/acceptor pair and a change in fluorescence is observed due to a
11 decrease in quenching. Cleavable donor/acceptor substrates have been
12 developed for chymotrypsin (*Li et al.* Bioconj. Chem., 10: 241-245 (1999)),
13 aminopeptidase P (*Hawthorne et al.*, Anal. Biochem., 253: 13-17 (1997)),
14 stromelysin (*Bickett et al.*, Ann. N. Y. Acad. Sci., 732: 351-355 (1994)) and
15 leukotriene D₄ hydrolase (*White et al.*, Anal. Biochem., 268: 245-251
16 (1999)). A chemosensor was described where binding of the ligand separates
17 the donor/acceptor pair (*Rothman et al.* Biorg. Med. Chem. Lett., 9: 509-512
18 (1999)).

19 In United States Patent No. 5,801,155 it was disclosed that
20 oligonucleotides (ODNs) having a covalently attached minor groove binder
21 (MGB) are more sequence specific for their complementary targets than
22 unmodified oligonucleotides. In addition the MGB-ODNs show substantial
23 increase in hybrid stability with complementary DNA target strands when
24 compared to unmodified oligonucleotides, allowing hybridization with shorter
25 oligonucleotides.

26 Reagents for fluorescent labeling of oligonucleotides are critical for
27 efficient application of the FRET assays described above. Other applications
28 such as DNA micro arrays also use fluorescently labeled DNA probes or
29 primers, and there is a need for improved reagents which facilitate synthesis
30 of fluorescent DNA. In general, phosphoramidite reagents and solid supports

1 are widely used on ODN synthesis. However, in the state of the art there are
2 not many commercially available phosphoramidite reagents for introducing
3 fluorescent groups into ODNs.

4 Linker groups to attach different ligand groups to ODNs play an
5 important role in the synthesis of oligonucleotide conjugates. A method for
6 the synthesis of 3'-aminohexyl-tailed oligonucleotides (*Petrie et al.*, *Bioconj.*
7 *Chem.*, 3: 85-87 (1992)), the use of a trifunctional trans-4-hydroxy-L-prolinol
8 group (*Reed et al.*, *Bioconjug. Chem.*, 2: 217-225 (1991)), diglycolic acid
9 (*Pon et al.*, *Nucl. Acids. Res.*, 25: 3629-3635 (1997)), 1,3-diol reagents (US
10 5,942,610 and US 5,451,463) and the non-nucleotide trifunctional reagent
11 (US 5,696,251) have been reported.

12 Resorufin and coumarin derivatives have been extensively used as
13 enzyme substrates to differentiate isozymes of cytochrome P450 (*Haugland et*
14 *al.*, *Handbook of Fluorescent Probes and Research Chemicals*, Six Edition,
15 Eugene, OR. pp. 235-236. 1996.). Reactive resorufin analogs have been
16 disclosed in United States Patent No. 5,304,645. Activated esters of coumarin
17 derivatives are known in the art (*Hirshberg et al.*, *Biochem.*, 37: 10391-5
18 (1998)). Coumarin-labeled dUTP incorporated in probes were used for *in situ*
19 hybridizations (*Wiegant et al.*, *Cytogenet. Cell Genet.*, 63: 73-76 (1993)).
20 Phosphoramidites to introduce labels into oligonucleotides have been
21 described in United States Patent Nos. 5,328,824 and 5,824,796.

22 Many current hybridization applications, require more than one
23 reporter molecule. In addition although reporter fluorophores are available to
24 be used in reporter/quencher pairs, most suffer from having some undesirable
25 characteristic, mixtures difficult to separate, positively charged, difficult to
26 synthesize, unstable during oligonucleotide synthesis or having overlapping
27 emission wavelengths with other desirable reporters. The present invention
28 provides reagents for oligonucleotide probes that address these unfavorable
29 characteristics and overcome some or all of the difficulties.

30

SUMMARY OF THE INVENTION

The present invention provides quencher molecules based on the 4-[4-nitrophenyl)diazinyl]phenylamine and/or the 4-[4-nitrophenyl)diazinyl]-naphthylamine structure. The quencher molecules have improved UV spectral overlap not only with commonly used fluorescent reporter groups that emit short wavelength range (about 400 to 500 nm), but have extended the range to the mid (525 nm = green) to long (670 nm = red) wavelengths. The quencher chromophores of the present invention are non-fluorescent, easily incorporated into DNA synthesis reagents, stable during automated DNA synthesis and during storage and compatible with no adverse effects on hybridization properties. Moreover, improved signal to noise ratios are observed with the fluorescent reporter dyes over a more extended wavelength range. Thus the present invention offers considerable advantages over the use of dabcyI (Nazerenko et al, Nucl. Acids Res., 25: 2516-21 (1997)) as a quenching dye, as used in the prior art.

In accordance with one aspect of the present invention the quenchers based on the 4-[4-nitrophenyl)diazinyl]phenylamine (and/or the 4-[4-nitrophenyl)diazinyl]naphthylamine structure) are modified with linker structures that allow their easy incorporation into fluorogenic DNA probes during automated DNA synthesis. The invention includes synthesis of phosphoramidites derived from the novel quencher molecules for incorporation of the quencher moieties into oligonucleotides during automated synthesis, and also synthesis of reagents derived from the novel quencher molecules for post solid-phase support attachment to amino-tailed oligonucleotides. In a related aspect, the novel quencher molecules are introduced into oligonucleotides using pyrazolo-[5,4-d]pyrimidines and pyrimidines phosphoramidites containing the quenchers attached at the 3'- and 5'-positions, respectively.

In accordance with another aspect of the invention, three different

1 fluorescent reagent types that are compatible with DNA synthesis are
2 synthesized or selected and converted into phosphoramidite reagents suitable
3 for incorporation onto ODNs. Specifically, violet fluorescent dyes based on
4 the 10-phenyl-1,3,5,7,9,10-hexahydropyrimidino[5',4'-5,6]pyridino[2,3-
5 d]pyrimidine-2,4,6,8-tetraone (PPT) structure, red fluorescent dyes based on
6 7-hydroxyphenoxazin-3-one (resorufin) and blue fluorescent dyes based on
7 the structure of coumarin are incorporated into phosphoramidite reagents.
8 These fluorescent dyes have excellent properties for multicolor fluorescent
9 analysis in combination with other dyes (eg. fluorescein). These reagents are
10 valuable for a variety of analytical methods that use either direct detection of
11 fluorescence or FRET detection formats. In a related aspect of the invention
12 the PPT-, coumarin- and resorufin-based fluorophores (fluorescent dyes) are
13 converted into novel reagents suitable for "post-oligonucleotide-synthesis"
14 covalent attachment at the 5'-end of ODNs. In another aspect, the new
15 fluorescent dyes are incorporated into oligonucleotides using pyrazolo-[5,4-
16 d]pyrimidines and pyrimidines phosphoramidites which contain the
17 fluorophores attached at the 3- and 5-positions, respectively.

18 In accordance with still another aspect of the invention, ODNs
19 covalently linked with the novel quencher structures of the invention, paired
20 with a covalently attached fluorescent moieties, are prepared. The resulting
21 FL-ODN-Q conjugate may also include a minor groove binder (MGB) that
22 improves the binding and discrimination characteristics of the resulting FL-
23 ODN-Q-MGB conjugate in diagnostic assays, particularly in the TaqMan
24 PCR assay of single nucleotide polymorphism (and the like) where allele-
25 specific discrimination not only requires probes with different fluorescent
26 reporter molecules but efficient quenchers. The quenchers used in accordance
27 with the invention in the FL-ODN-Q-MGB conjugates provide broad
28 quenching wavelength range, and certain novel reporter labeling reagents in
29 accordance with the invention have distinctive emission wavelengths for
30 improved multicolor analysis.

1 In one application of the principles summarized above, fluorogenic
2 probes are prepared using a universal "3'-hexanol" solid support (available in
3 accordance with *Gamper et al. Nucleic Acids Res.*, 21: 145-150 (1993)
4 expressly incorporated herein by reference), where a quencher
5 phosphoramidite of the invention is added at the first coupling step (3'-end)
6 of the ODN sequence and a fluorophore (FL) was attached at the final
7 coupling step, yielding 5'-FL-ODN-Q-hexanol conjugate probes.

8 As other applications of the invention, methods for synthesizing and
9 attaching the novel quenchers to ODN-fluorophore conjugates with and
10 without a 3'-minor groove binder (MGB) are disclosed. These methods
11 utilize synthetic solid supports for automated oligonucleotide synthesis with
12 cleavable linkers.

13 In another application, a fluorogenic oligonucleotide probe is prepared
14 from a MGB modified solid support substantially in accordance with the
15 procedure of *Lukhtanov et al. Bioconjugate Chem.*, 7: 564-567 (1996),
16 where a quencher- phosphoramidite of the invention is added at the first
17 coupling step to the MGB, and a fluorophore (FL) is attached at the final
18 coupling step to the ODN, to yield 5'-FL-ODN-Q-MGB conjugate probe.

19 Additional application of the methods and compositions of the present
20 invention are in micro-arrays in nucleic acid-based diagnostic assays which
21 recently have become important in many fields, such as the medical sciences,
22 forensics, agriculture and water quality control. Other related application of
23 the methods and compositions of the present invention are in procedures
24 using arrays of oligonucleotides, such as the array-based analysis of gene
25 expression (*Eisen, Methods of Enzym.*, 303: 179-205 (1999)). In these
26 procedures, an ordered array of oligonucleotides or DNAs that correspond to
27 all, or a large fraction of the genes in many organism is used as a platform for
28 hybridization. Microarray-based methods are used in assays to measure the
29 relative representation of expressed RNA species. The quantitation of
30 differences in abundance of each RNA species is achieved by directly

1 comparing two samples by labeling them with spectrally distinct fluorescent
2 dyes and mixing the two probes for simultaneous hybridization to one array.

3 To the extent the application of the compositions and methods of
4 present invention relates to the detection of nucleic acids, it includes but is not
5 limited to methods where FRET is involved, such as 5'-nuclease, universal
6 energy transfer primers or beacon assays. These methods are usually directed
7 to, but are not limited to the detection of PCR-generated nucleic acid
8 sequences. Some of these methods involve simultaneous detection of more
9 than one nucleic acid sequence in the same assay. Similarly, the invention
10 relates to methods where FRET is involved in the detection of protein
11 concentration or enzyme activity.

12 Still other applications of the invention relate to the labeling with
13 luminescent PPT-, coumarin- and resorufin-based dyes of nucleic acids,
14 proteins and other materials including, drugs, toxins, cells, microbial
15 materials, particles, glass or polymeric surfaces and the like, at a reactive
16 group such as an amino, hydroxyl or sulfhydryl group. The present invention
17 may be used in single- and two-step labeling processes. In the two-step
18 labeling process, a primary component, such as an oligonucleotide is labeled
19 with the reagent capable of introducing the novel fluorophore PPT-,
20 coumarin- and resorufin-based dyes, by reaction with a reactive group of the
21 ODN (such as an amine, hydroxyl, carboxyl, aldehyde or sulfhydryl group)
22 and the label is used to probe for a secondary component, such as an
23 oligonucleotide target.

24

25 BRIEF DESCRIPTION OF THE DRAWINGS

26

27 **Figure 1** is a schematic representation of real-time 5'-nuclease PCR
28 assay.

29 **Figure 2** is a graph showing the UV spectra of Dabcyl- and Red13
30 dye-modified DNA probes.

1 **Figure 3** is a graph showing the performance of fluorogenic MGB
2 probes in a "real-time" PCR assay.

3 **Figure 4** is a graph showing the fluorescent spectra of violet, FAM
4 and resorufin dye containing DNA probes .

6 **DETAILED DESCRIPTION OF INVENTION**

8 **QUENCHER REAGENTS FOR OLIGONUCLEOTIDE SYNTHESIS**

9 Two types of reagents for introducing the substituted 4-
10 (phenyldiazenyl)phenylamine quencher moieties into oligonucleotides using
11 an automated DNA synthesizer are exemplified in the disclosure below. Here
12 and in the reaction schemes the abbreviations MGB, FL, Q, CPG and ODN
13 stand for "minor groove binder", "fluorescent or fluorophor", "quencher"
14 "controlled pore glass" and "oligonucleotide" moieties or molecules,
15 respectively, and in a manner which is apparent from context.

16 Dimethoxytrityl Protected Quencher Phosphoramidites

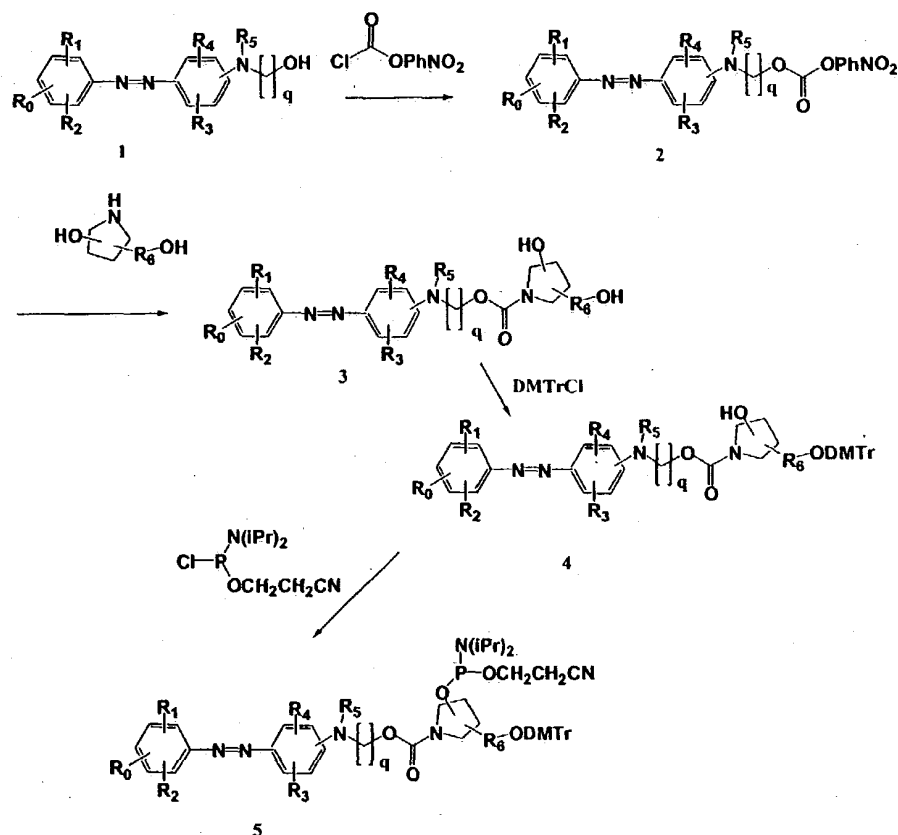
17 The first type of reagents disclosed herein are phosphoramidites that
18 bear the quencher molecule (Q) as well as a dimethoxytrityl (DMTr)
19 (methoxytrityl, trityl or the like acid labile blocking group) protected primary
20 alcohol that provides an attachment point for the growing
21 oligodeoxynucleotide (ODN) chain during subsequent oligonucleotide
22 synthesis. Examples of these reagents are depicted in **Formulas 1, 2, and 3**,
23 and in **Reaction Schemes 1 and 2**.

24 In **Reaction Scheme 1** the starting compound is a substituted 4-
25 (phenyldiazenyl)phenylamine **1** that has a primary hydroxyl group. Such
26 starting material is commercially available or can be synthesized in
27 accordance with methods known in the art, applying routine skill available to
28 the practicing organic chemist. For example 4-nitrobenzondiazonium salt is
29 reacted with 2-(2-chloroanilino)ethanol to yield 2-[2-chloro-4-(4-
30 nitrophenylazo)anilino]ethanol in accordance with the teachings of United

1 States Patent No. 2,264,303. 2-[2-chloro-4-(4-nitrophenylazo)anilino]ethanol
2 is within the scope of compound **1** as depicted in **Reaction Scheme 1**. The
3 specification of United States Patent No. 2,264,303 is expressly incorporated
4 herein by reference.

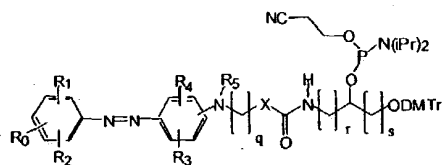
5 Other examples of commercially available starting materials (or of
6 their precursors) are: 2-(ethyl{4-[(4-nitrophenyl)diazenyl]phenyl}amino)-
7 ethan-1-ol and 2-(ethyl{4-[(2-methoxy-4-nitrophenyl)diazenyl]phenyl}-
8 amino)ethan-1-ol.

9 As is shown in **Reaction Scheme 1**, compound **1** is reacted with p-
10 nitrophenylchloroformate to yield the carbonate **2**. Reaction of **2** with
11 substituted pyrrolidinediols yields a diol intermediate **3**. The pyrrolidinediol
12 is a trifunctional reagent that has an amino, a primary and a secondary
13 hydroxyl group. An example of a pyrrolidinediol as well as examples of other
14 trifunctional reagents having an amino, primary and a secondary hydroxyl
15 group, are described in United States Patent No. 5,512,667 the specification
16 of which is incorporated herein by reference. The diol **3** is reacted first with
17 dimethoxytrityl chloride (DMTrCl) to block the primary hydroxyl group of
18 the trifunctional reagent and yield intermediate **4**. The intermediate **4**, still
19 having a free secondary hydroxyl group in the trifunctional reagent, is then
20 reacted with 2-cyanoethyl diisopropylchlorophosphoramidite to give the
21 dimethoxytrityl protected phosphoramidite reagent **5**. In the compounds
22 shown in **Reaction Scheme 1** the symbols are defined as follows. R_0 , R_1 , R_2 ,
23 R_3 and R_4 are independently -H, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$ where
24 $n=0$ to 5 , $-NO_2$, $-SO_3$, $-N[(CH_2)_{n'}CH_3]_2$ where $n'=0$ to 5 or $-CN$; $R_5=-H$ or -
25 $(CH_2)_{n''}CH_3$ where $n''=0$ to 5 ; $R_6=-(CH_2)_{n^*}$ where $n^*=1$ to 5 and $q=1$ to 20 .
26 The dimethoxytrityl protected phosphoramidite reagent **5** is suitable for
27 attachment to oligonucleotides in steps otherwise known in routine ODN
28 synthesis.

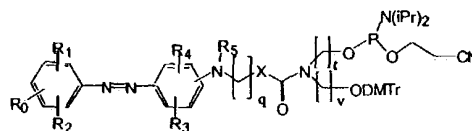


REACTION SCHEME 1

In other embodiments, using the reactions described in Reaction Scheme 1, (or only such modifications which are within the skill of the practicing organic chemist) starting with other trifunctional reagents having an amino and two hydroxyl groups, the phosphoramidites of Formula 1 and Formula 2 are synthesized, where q, R₀, R₁, R₂, R₃, R₄ and R₅, are defined as above; r and s independently are 1 to 20; X is -O- or -CH₂-; t and v independently are 1 to 20.

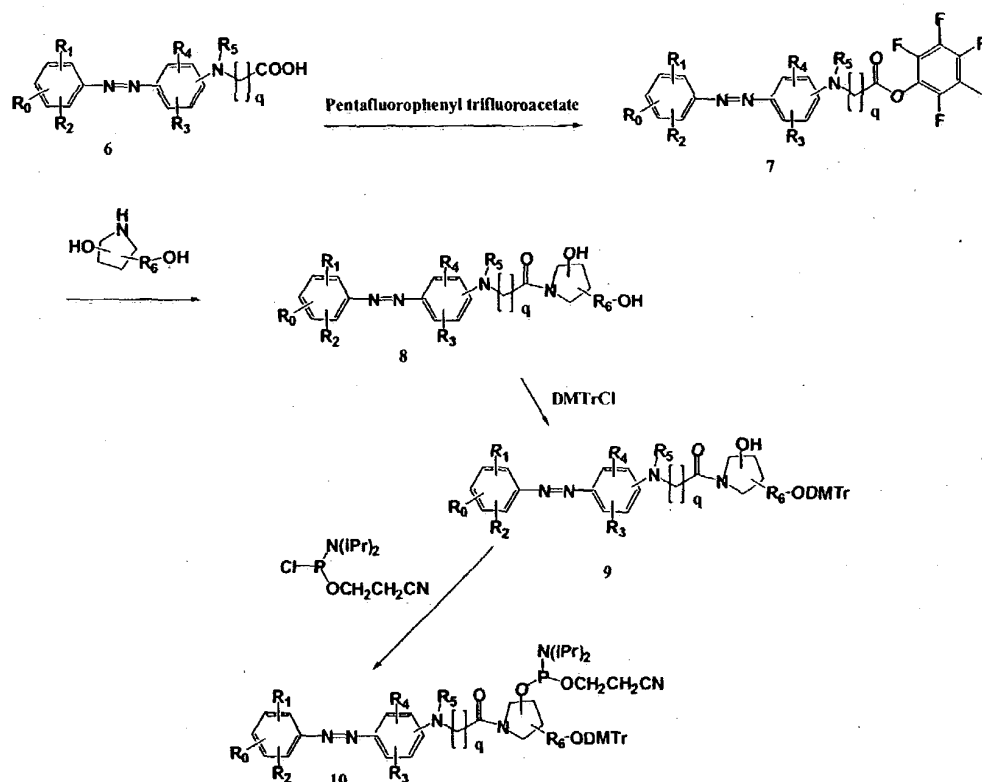


Formula 1



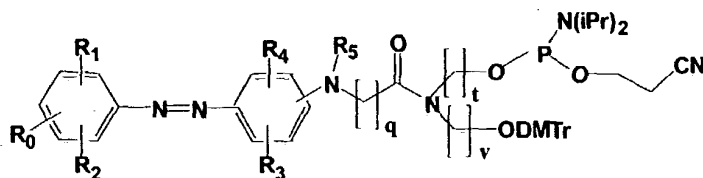
Formula 2

1 **Reaction Scheme 2** discloses the synthesis of another exemplary
2 phosphoramidite reagent **10** bearing the substituted 4-(phenyldiazenyl)-
3 phenylamine quencher moiety and including the trifunctional pyrrolidinediol
4 moiety. In this synthetic scheme the starting material is a substituted 4-
5 (phenyldiazenyl)phenylamine compound **6** that has a free carboxyl group.
6 Compound **6** (commercially available or made in accordance with the
7 chemical literature within the skill of the practicing organic chemist) is
8 reacted with pentafluorophenyl trifluoroacetate to make an active ester **7**,
9 which is thereafter reacted to couple the substituted 4-(phenyldiazenyl)-
10 phenylamine moiety to the ring nitrogen of a pyrrolidinediol moiety having a
11 free primary and a free secondary hydroxyl group, yielding compound **8**.
12 Treatment of **8** with DMTrCl followed by reaction with 2-cyanoethyl
13 diisopropylchlorophosphoramidite gives the dimethoxytrityl protected
14 phosphoramidite reagent **10**. In **Reaction Scheme 2** the symbols are defined
15 the same as in **Reaction Scheme 1**.



REACTION SCHEME 2

In still another example, using the reactions described in **Reaction Scheme 2**, starting with a substituted 4-(phenyldiazenyl)phenylamine (compound **6**) and using a non-cyclic trifunctional reagent (having an amino and two hydroxyl functions) instead of the pyrrolidinediol shown in **Scheme 2**, the dimethoxytrityl protected phosphoramidite of **Formula 3** is synthesized, where q , R_0 , R_1 , R_2 , R_3 , R_4 , R_5 , and R_6 are defined as above and t and v independently are 1 to 20.

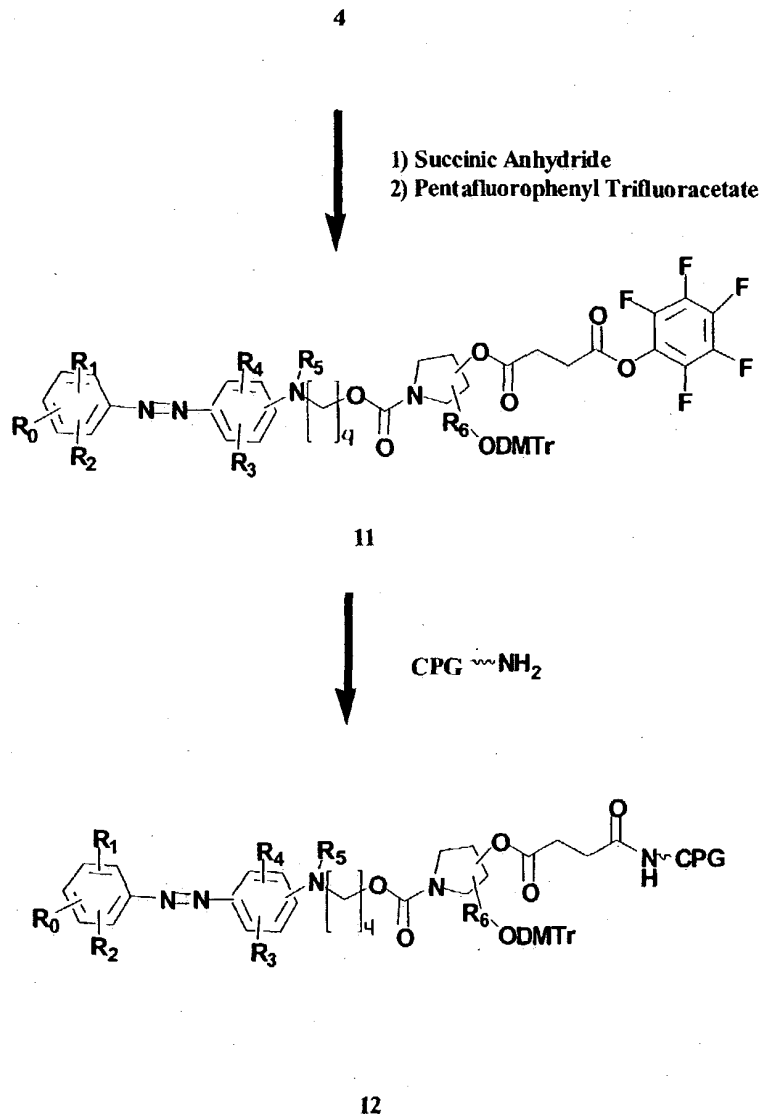


Formula 3

1

2 Quenchers Attached to Solid Support through (or similarly) Protected Linker,
3 Suitable for ODN Synthesis

4 A second class of compounds or reagents suitable for introducing the
5 quencher molecules into ODNs constitute a composition that has a solid
6 support of the type used for ODN synthesis (for example controlled pore glass
7 (CPG)), and linker attaching the quencher to the solid support. The linker
8 has a hydroxyl function that is protected, usually by a dimethoxytrityl group
9 which is removed during the synthesis when the first nucleotide is attached to
10 the linker. Generally speaking the same quencher/linker intermediates
11 described above in **Reaction Scheme 1** can also be used to prepare these
12 reagents (CPG beads) having the exemplary structure **12**, shown in **Reaction**
13 **Scheme 3**.

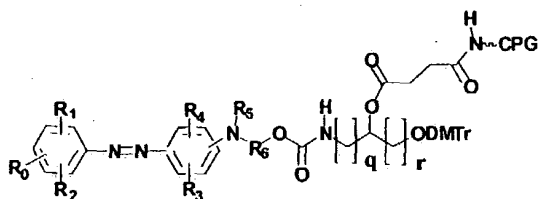


REACTION SCHEME 3

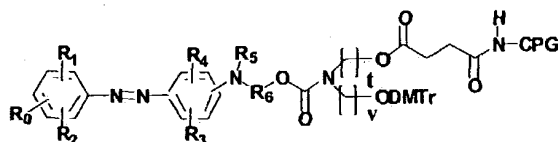
In accordance with this scheme, the secondary hydroxyl group of the intermediate 4 (shown in Scheme 1) is reacted with succinic anhydride, and thereafter pentafluorophenyl trifluoroacetate to provide the active ester 11. The active ester 11 is then reacted with the free amino group attached to the solid support (CPG bead) to provide the modified solid support 12. Whereas the exemplary modified solid support 12 includes the “trifunctional linker”

1 derived from pyrrolidine diol, it will be readily understood by those skilled in
 2 art that analogous modified solid supports including other linkers and related
 3 structures, such as the linkers shown in **Formulas 1, 2 and 3** can also be made
 4 substantially in accordance with **Reaction Scheme 3**, resulting in modified
 5 solid support compositions including the quencher moiety, such the ones
 6 shown in **Formula 4** and **Formula 5**.

7 The modified solid support compositions including the quencher
 8 moiety of structure **12** and of **Formula 4** and **5** are used for preparing 3'-
 9 quencher conjugates, allowing the introduction of a fluorophore at the 5'-end
 10 with the appropriate phosphoramidite, or post-synthetically with a
 11 fluorophore containing a reactive group. In **Reaction Scheme 3** and in
 12 **Formula 4** and **Formula 5** the symbols are defined as above. It should be
 13 understood that other solid supports (such as polystyrene) and other cleavable
 14 linker systems (in addition to the succinate linker shown) can also be prepared
 15 in accordance with these generic teachings and therefore are also within the
 16 scope of the invention.



Formula 4



Formula 5

19 Minor Groove Binder Quencher Reagents for Oligonucleotide Synthesis

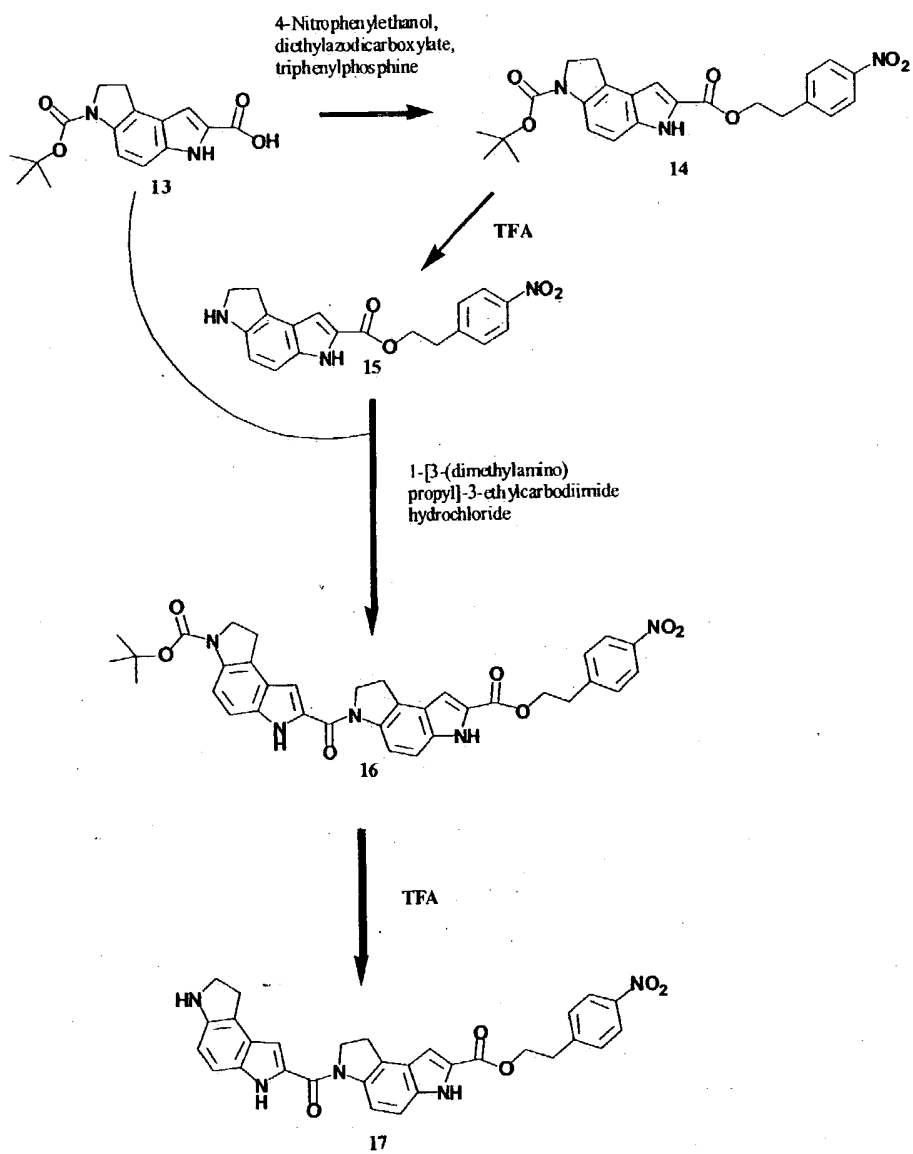
20 In one embodiment a minor groove binder (MGB) is attached to
 21 controlled pore glass (CPG) through a cleavable linker. A quencher moiety,

1 based on the 4-(phenyldiazenyl)phenylamine structure, is attached through a
2 linker molecule to the MGB. The linker molecule also contains a hydroxyl
3 group blocked with DMTr (or like) blocking group. After removal of the
4 DMTr group, an oligonucleotide is synthesized on an automated
5 oligonucleotide synthesizer by step-wise attachment of nucleotide units to the
6 hydroxyl group. A fluorophore is introduced at the 5'-end with the
7 appropriate phosphoramidite, or post-synthetically with a fluorophore
8 containing a reactive group, to yield an ODN having an attached fluorescent
9 moiety (FL), quencher (Q) and MGB (FL-ODN-Q-MGB). In this connection
10 it is noted that the synthesis of MGBs and their attachment to ODNs is well
11 known (see for example United States Patent No. 5,801,155, 09/539,097 and
12 09/141,764; all of which are expressly incorporated herein by reference.

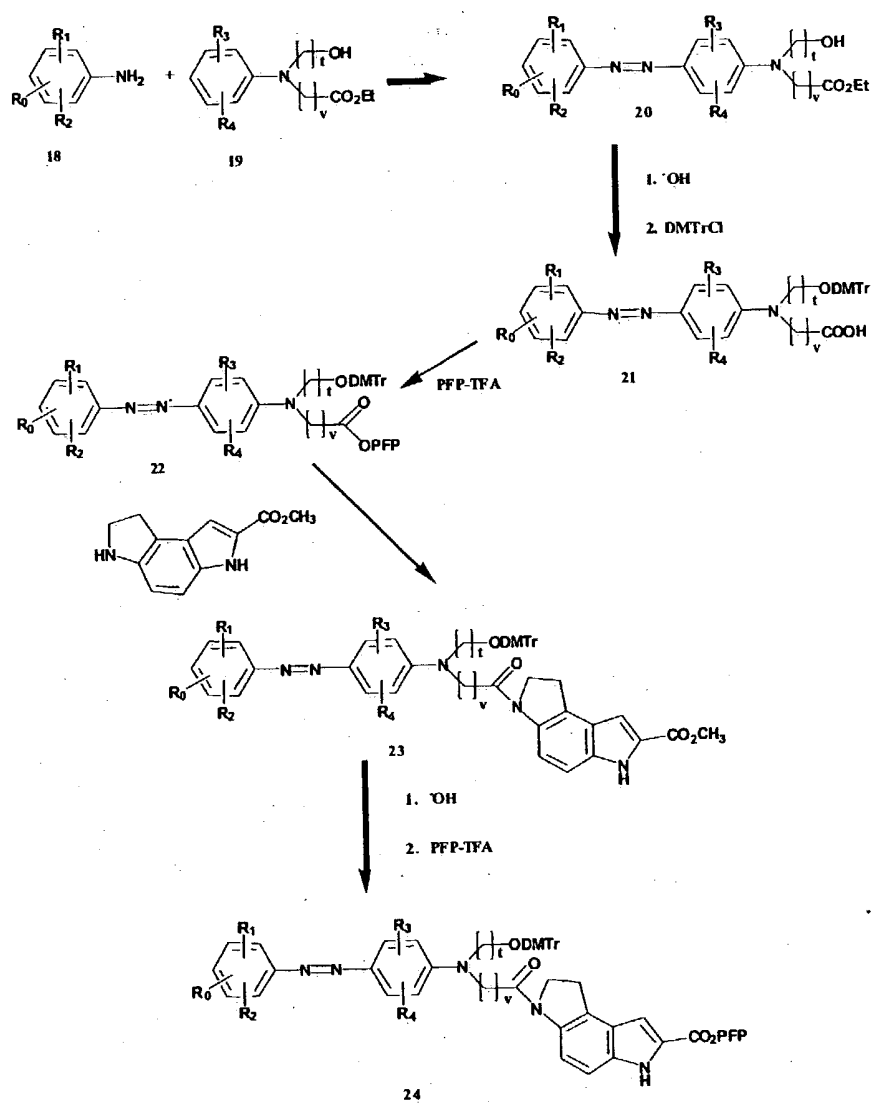
13 In a preferred embodiment the MGB is 3-{{3-(pyrrolo[4,5-e]indolin-7-
14 ylcarbonyl)pyrrolo[4,5-e]indolin-7-yl}carbonyl}pyrrolo[3,2-e]indoline-7-
15 carboxylic acid (DPI₃). The synthesis of the covalently bound "aggregate"
16 FL-ODN-Q-DPI₃ requires five phases, described below. The first phase,
17 shown in **Reaction Scheme 4**, is the synthesis of an intermediate, 2-(4-
18 nitrophenyl)ethyl 3-(pyrrolo[4,5-e]indoline-7-carbonyl)pyrrolo[4,5-
19 e]indoline-7-carboxylate (DPI₂-NPC) **17**. The second phase, shown in
20 **Reaction Scheme 5**, is the synthesis of Q-DMTr-DPI-CO₂PFP **24** where a
21 quencher is coupled through a linker to a pyrrolo[3,2-e]indoline-7-carboxylic
22 acid unit (DPI). Here, and in the reaction schemes PFP stands for the
23 pentafluorophenyl or pentafluorophenyl group, as the context requires.
24 In the third phase, shown in **Reaction Scheme 6**, DMTr-Q-DPI₃-PFP **25a** is
25 synthesized from **17** and **24**. In the fourth phase **25a** is coupled to CPG to
26 yield a DMTr-Q-DPI₃-CPG **29**, and in the fifth phase **29** is used on an
27 automated oligonucleotide synthesizer to stepwise attach nucleotide units and
28 to provide, after removal from the CPG, the product FL-5'-ODN-3'-Q-DPI₃
29 **30**.

30 The fourth and fifth phases of these synthetic process are shown in

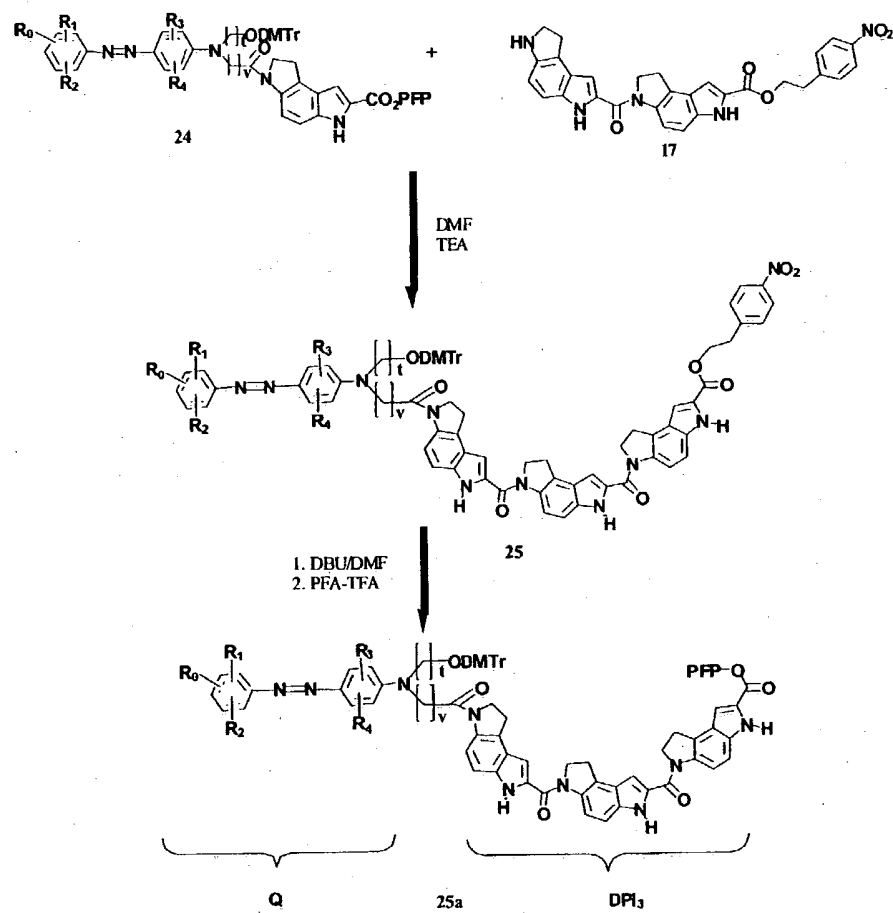
- 1 **Reaction Scheme 7.** Experimental conditions for this sequence (phases 1
- 2 through 5) are described below.



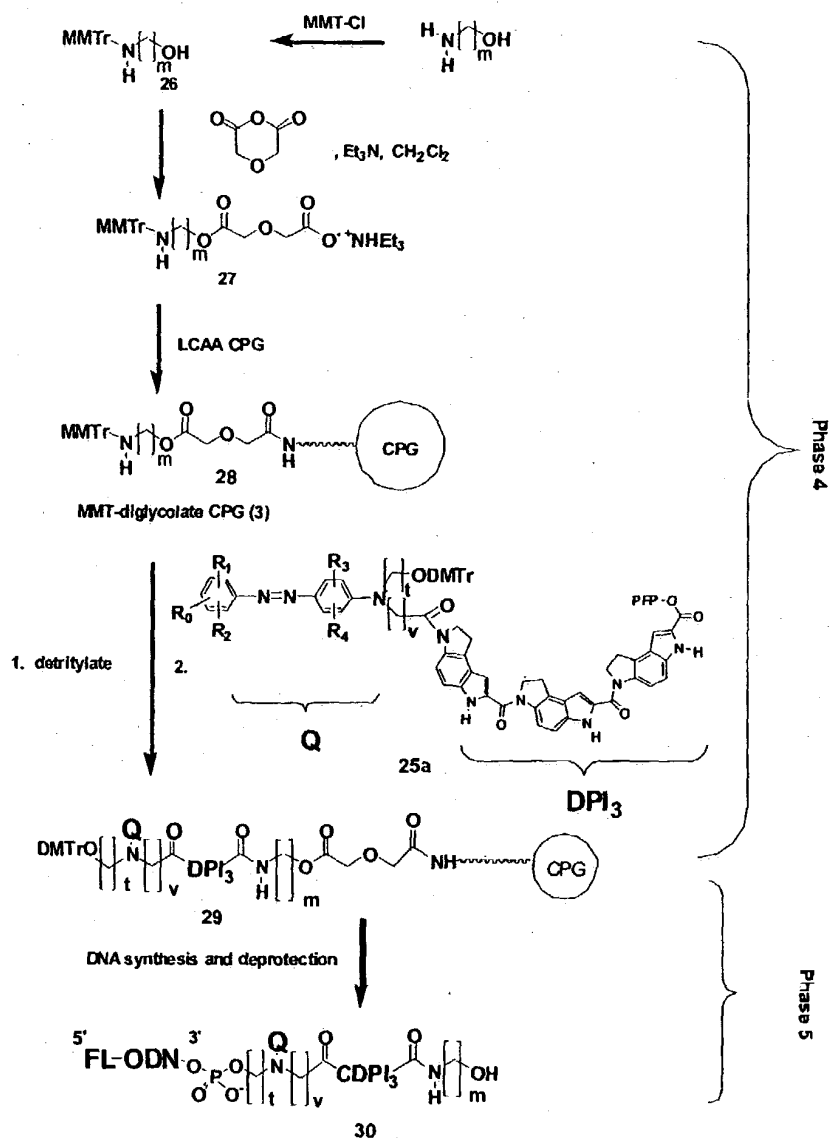
REACTION SCHEME 4



REACTION SCHEME 5



REACTION SCHEME 6



REACTION SCHEME 7

Describing these phases or reactions now in more detail, Q-DPI₃ moiety **25** (phase 3) is synthesized by the reaction of two intermediates, **17** and **24** as shown in Reaction Scheme 6. The first intermediate DPI₂-NPE **17** is made as shown in Scheme 4. DPI-tBoc **13** was reacted with p-nitrophenylethanol in the presence of diethylazodicarboxylate (DEAD) and triphenylphosphine to yield the di-ester **14**. Compound **14** was first treated with trifluoroacetic acid (TFA) to yield **15**, and then conjugated with **13** in

1 the presence of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide
2 hydrochloride to give the 4-nitrophenyl ester of DPI₂ **16** in good yield.
3 Reaction of **16** with TFA gives the p-nitrophenethyl ester of DPI₂ **17**. The
4 second intermediate DPI-Q **24** (phase 2) is synthesized as shown in **Reaction**
5 **Scheme 5**. A substituted nitroaniline **18** (available commercially or in
6 accordance with the chemical literature) is diazotized in the presence of
7 nitrous acid and is coupled to a substituted aniline **19** (available commercially
8 or in accordance with the chemical literature) to form the azo intermediate
9 quencher molecule **20**. Alkaline hydrolysis of the ethyl ester **20** followed by
10 the treatment with DMTrCl gives the DMTr-Q **21**, that is subsequently
11 activated with pentafluorophenyl trifluoroacetate to yield **22**. Reaction of **22**
12 with DPI-methyl ester gives the Q-DMTr-DPI methylester **23**. Compound **23**
13 is then treated with alkali to hydrolyze the methyl ester and then activated
14 with PFP-TFA to yield Q-DMTr-DPI PFPester **24**. In **Reaction Scheme 5**
15 the symbols R₀, R₁ through R₄, v and t are defined as above.

16 Referring now to **Reaction Scheme 6**, where the symbols are also
17 defined as above, DMTr-Q-DPI₃-PFP **25a** (third phase) is synthesized first
18 by reacting the activated quencher **24** (DMTr-Q-DPI PFP) with DPI₂-NPC **17**
19 to yield the p-nitrophenylethyl ester **25**, which is converted to the active ester
20 **25a**, first by treatment with base such as 1,8-diazabicyclo[5.4.0]undec-7-ene
21 (DBU) to remove the p-nitrophenylethyl moiety and then treatment with
22 2,3,4,5,6-pentafluorophenyl trifluoroacetate (PFP-TFA).

23 The synthesis of DMTr-Q-DPI₃-CPG **29** (phase four) is shown in
24 **Reaction Scheme 7**. In this synthetic sequence the improved quencher
25 molecule becomes attached through a cleavable diglycolate linker to
26 controlled pore glass beads (CPG). Specifically, aminopropanol, or a
27 homolog thereof, is reacted successively with monomethoxytrityl chloride
28 (MMTr-Cl) and then with diglycolic anhydride to form MMT-blocked
29 aminopropanol **26** (or homolog) and MMT-diglycolate **27**, respectively. The
30 symbol **m** is defined as an integer having the values 2 to 20. For the presently

1 preferred aminopropanol **m** is 3. The remaining symbols in this scheme are
2 defined as above. Reaction of **27** with long chain aminoalkyl CPG in the
3 presence of activating agents (HOBT and HBTU), yields the MMT-
4 diglycolate-CPG **28**, that is converted after detritylation and reaction with **25a**
5 to DMTrO-Q-DPI₃-CPG **29**.

6 In phase 5, still shown in **Reaction Scheme 7** oligonucleotide
7 synthesis is performed with the aid of an automated DNA synthesizer, and a
8 fluorophore is attached at the 5'-end of the ODN, using either a fluorophore-
9 phosphoramidite or a fluorophore containing a reactive group, to yield the FL-
10 ODN-Q-DPI₃ **30** conjugate.

11 The FL-ODN-Q-DPI₃ **30** conjugate can also be synthesized by an
12 alternative synthetic route which is not specifically illustrated in the reaction
13 schemes. In this alternative route DPI₃-methyl ester (obtained in accordance
14 with *Boger et al.*, J. Org. Chem., 52: 1521- (1987) incorporated herein by
15 reference) is first reacted with compound **22** and then with alkali to give Q-
16 DPI₃-methyl ester and Q-DPI₃-COOH, respectively. The latter compound is
17 then activated with pentafluorophenyl trifluoroacetate, to yield **25a**, which is
18 then used in the reactions shown in **Scheme 7**, to yield **30**.

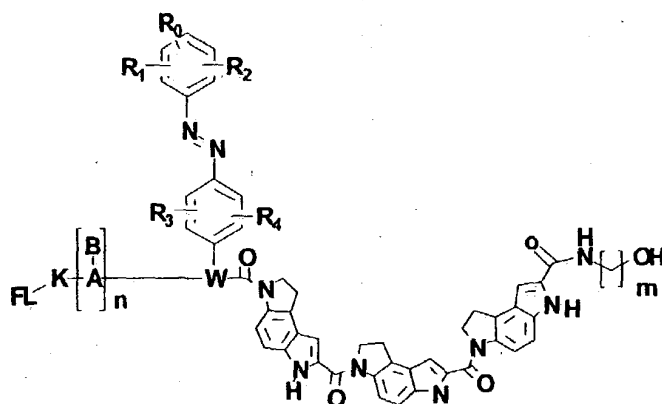
19 FL-ODN-Q and FL-ODN-Q-MGB Probes

20 A general structure of a preferred embodiment of FL-ODN-Q-DPI₃
21 conjugates is shown in **Formula 6** where:

22 FL is a fluorophore with emission wavelengths in the range of about
23 300 to about 800 nm and more preferably 400 to 700 nm; K is a linker
24 containing between 1 and 30 atoms, which include any of C, O, N, S, P and
25 H; [A-B]_n symbolizes a DNA, RNA or PNA or any combination thereof,
26 where A is the sugar phosphate backbone (including modified sugars and
27 modified phosphates), B is the heterocyclic base, and n is the number of
28 nucleotide units. B can independently be any of the purine- and pyrimidine-;
29 pyrazolo[3,4-d]pyrimidine-, 7-substituted pyrazolo[3,4-d]pyrimidine-, 7-
30 deazapurines, 7-substituted 7-deazapurines, modified purine- and pyrimidine-

1 bases, and the oligonucleotide or nucleic acid can include any combinations
 2 of these bases. W is a linker of the length of 0 to approximately 30 atoms,
 3 selected from the group consisting of C, O, N, S, P and H, furthermore W is a
 4 substituted branched aliphatic chain, or a substituted ring structure or a
 5 combined substituted aliphatic and ring structure; R_0 , R_1 , R_2 , R_3 and R_4 are as
 6 described previously and $m=1$ to 20.

7



8

Formula 6

9

10 Syntheses of PNA and PNA/DNA chimeras are known in the art and
 11 can generally speaking be performed in accordance with the publications
 12 *Uhlmann et al.*, Angew. Chem. Inter. Ed., 37:2796-2823 (1998); *Mayfield et*
 13 *al.*, Anal. Biochem., 401-404 (1998) which are incorporated herein by
 14 reference.

15 A still more preferred embodiment within the scope of **Formula 6** is
 16 one where W is $-(CH_2)_3N(-)-(CH_2)_3-$; $R_0=NO_2$; $R_1=Cl$; $R_2=R_3=R_4=H$; K is a
 17 6 carbon linker and $m=3$.

18 Conjugate probes of the present invention containing a fluorescent
 19 reporter-quencher pair are, generally speaking, used in conjunction with the
 20 amplification of target polynucleotides, frequently in methods utilizing PCR,
 21 as described for example by *Holland et al.* Proc. Acad. Sci., 88: 7276-

1 7280(1991) and *Wittwer et al.*, *Biotechniques*, 22: 176-181 (1997) which are
2 incorporated herein by reference. The binding site of the conjugate probe is
3 located between the PCR primers used to amplify the target polynucleotide.

4 Use of the conjugate oligonucleotide probes according to the present
5 invention for detection of target oligonucleotide sequences provides several
6 advantages over prior-art reporter quencher groups and combinations. For
7 example, the quenchers including the 4-[4-nitrophenyl]diazinyl]phenylamine
8 structure in accordance with the present invention gave larger signal to noise
9 ratios (S/N) in probes with either FAM or TAMRA serving as reporters than
10 dabcyl as a quencher. Furthermore, the quenchers in accordance with the
11 invention show a broader absorbance range than dabcyl, allowing efficient
12 quenching of a broad range of fluorophores. In addition, in MGB-
13 oligonucleotide conjugates have improved hybridization characteristics, an
14 improved quencher showed about 30-fold increase in S/N ratio with TAMRA
15 compared to a standard probe (no DPI₃) with dabcyl. Reagents of the present
16 invention allow the introduction of the quencher during automated
17 oligonucleotide synthesis. (DabcyI phosphoramidite is commercially
18 available; (Glen Research, Sterling, VA))

19 It should be understood that, generally speaking, for the purpose of this
20 invention, an oligonucleotide comprises a plurality of nucleotide units, a 3'
21 end and a 5' end. The oligonucleotide may contain one or more modified
22 bases other than the normal purine and pyrimidine bases, as well as modified
23 internucleotide linkages capable of specifically binding target polynucleotide
24 through Watson-Crick base pairing, or the like. In addition, oligonucleotides
25 may include peptide oligonucleotides (PNAs) or PNA/DNA chimeras, the
26 synthesis of which is known and can be performed for example in accordance
27 with the publications *Uhlmann et al.*, *Angew. Chem. Inter. Ed.*, 37:2796-
28 2823 (1998) and *Mayfield et al.*, *Anal. Biochem.*, 401-404 (1998); all of
29 which are expressly incorporated herein by reference.

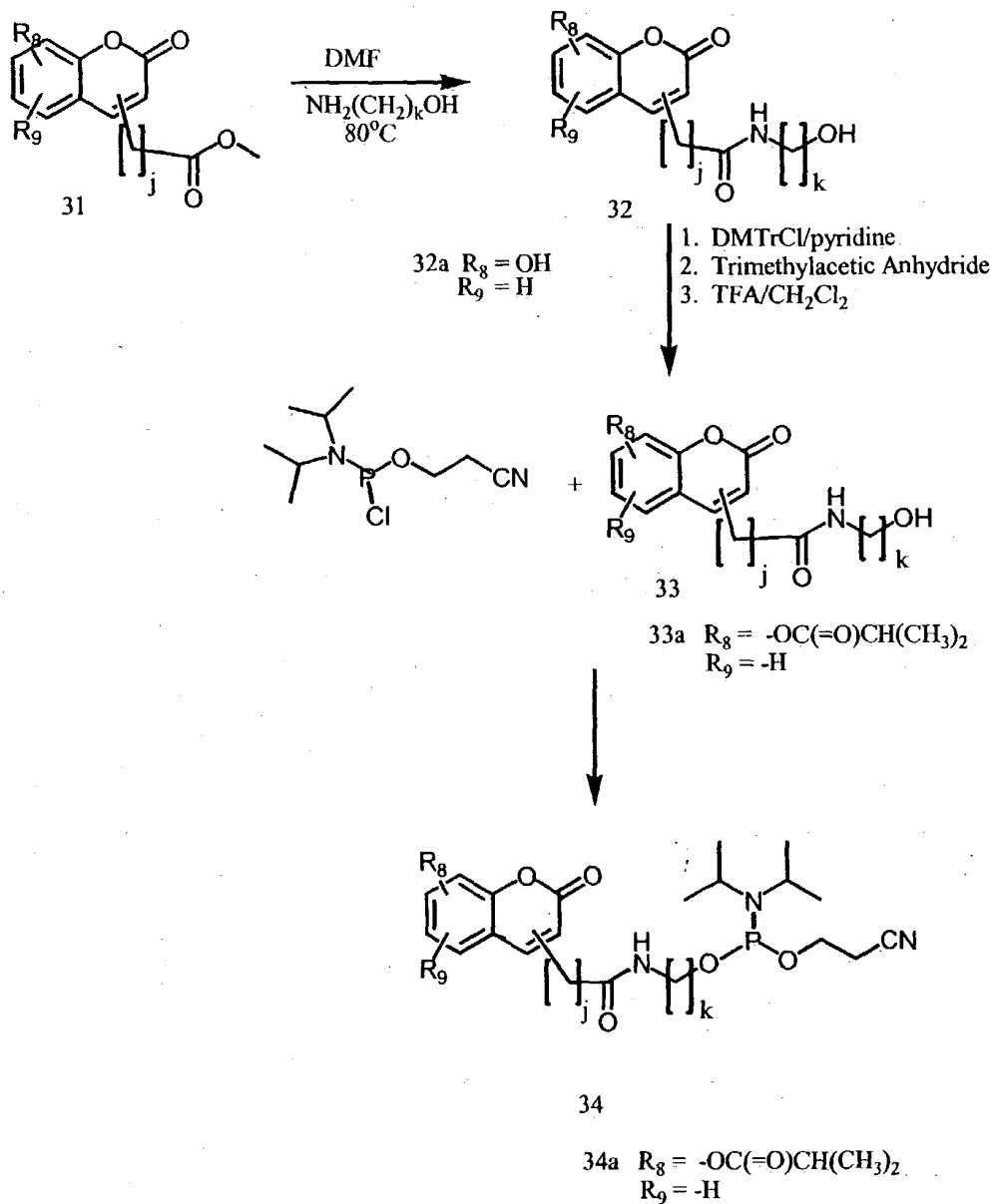
30 Generally, the oligonucleotide probes of the invention will have a

1 sufficient number of phosphodiester linkages adjacent to the 5' end to allow
2 5'-3' exonuclease activity to allow efficient cleavage between the quencher
3 and fluorophore molecules in the probe. An adequate number in this regards
4 is approximately between 1 and 100.

5 6 FLUOROPHORE REAGENTS

7 8 Coumarin Phosphoramidite Reagents

9 Fluorescent dyes which have emission wavelengths shorter than the
10 green fluorescent dye FAM also have utility in DNA probe based assays.
11 However in the prior art these probes have been less popular since excitation
12 with laser light sources is less feasible than with longer wavelengths. To the
13 best knowledge of the present inventors, to date, there have been no reports of
14 DNA synthesis reagents that contain blue fluorescent dyes. An example of
15 such a phosphoramidite reagent containing a preferred coumarin fluorophore
16 and which is suitable for DNA synthesis, is shown in **Reaction Scheme 8**, as
17 compound **34**. In the phosphoramidite reagent **34**, R_8 and R_9 independently
18 are H, halogen, $-\text{NO}_2$, $-\text{SO}_3$, $-\text{C}(=\text{O})\text{NH}_2$, or $-\text{CN}$; $-\text{OR}_{nn}$, $-\text{SR}_{nn}$, $-\text{OR}_{nn}$, $-\text{NHR}_{nn}$, $-\text{N}[\text{R}_{nn}]_2$ where R_{nn} is independently H, a blocking group compatible
19 with oligomer synthesis and which can be removed under acid or alkaline
20 conditions; or a group that contains between 1 and 10 carbon atoms, j and k
21 independently are 1 to 10. As can be seen the reagent **34** includes a
22 covalently linked coumarin chromophore which emits light at 458 nm. DNA
23 probes containing this coumarin chromophore were prepared and gave the
24 desired fluorescent emission properties.
25



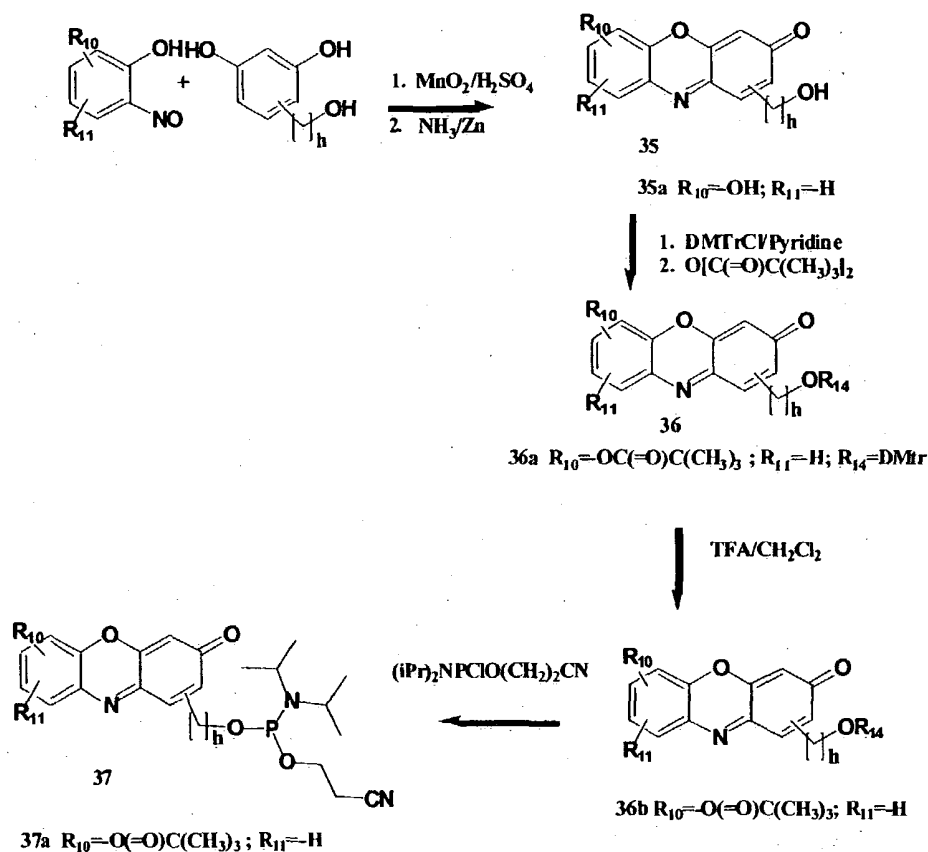
REACTION SCHEME 8

1
2

3 Referring now to **Scheme 8** in general terms and also in an example
 4 that provides the specific phosphoramidite reagent **34a**, a hydroxyl substituted
 5 (2-oxo-2H-chromen-4-yl)-alkylcarboxyl methyl ester (**31**) is obtained
 6 according to the publication *Baker et al.* (J.Chem.Soc.; 170, 173 (1950))
 7 incorporated herein by reference. Compound **31** is converted to the alcohol

1 derivative **32** (specifically to **32a** where R_8 is -OH and R_9 is -H) by reaction
 2 with an aminoalkanol at 80°C. Reaction of **32** first with DMTrCl and then
 3 with trimethylacetic anhydride followed by the removal of the DMTr
 4 blocking group gives a pivaloate derivative **33**, in the specific example **33a**
 5 where R_8 is -OC(=O)CH(CH₃)₂ and R_9 is -H. Reaction of **33** with 2-
 6 cyanoethyl diisopropylchlorophosphoramidite gives reagent **34** (specifically
 7 **34a** where R_8 is -OC(=O)CH(CH₃)₂, R_9 is -H). The reagent **34** is used for
 8 incorporating the coumarin fluorophore into the 5'-terminus of DNA probes.
 9 It is noteworthy that removal of the protecting groups during automated
 10 oligonucleotides synthesis proceeded well, resulting in high yields. The
 11 symbols **j** and **k** in **Scheme 8** are defined as 0 to 20 and 1 to 20, respectively.
 12 **Resorufin Phosphoramidite**

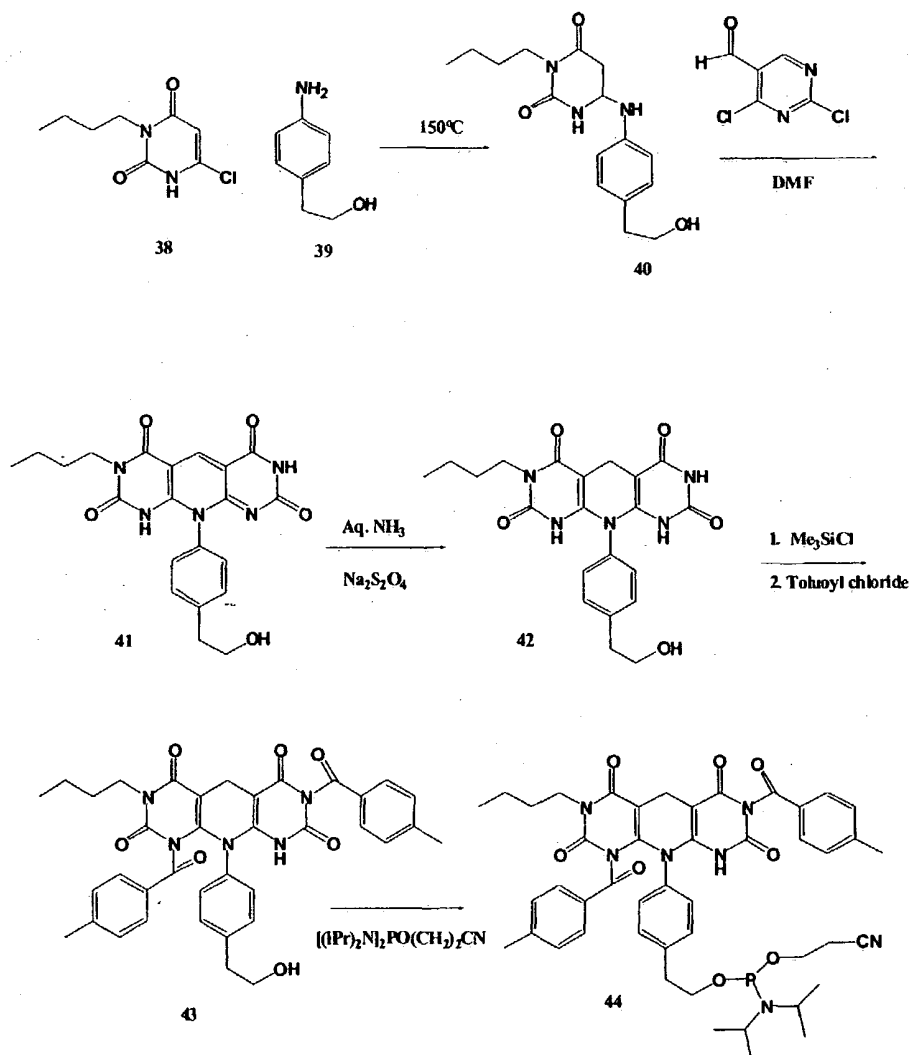
13 Another new class of DNA synthesis reagents are based on the 7-
 14 hydroxy-3H-phenoxazin-3-one chromophore present in the parent compound
 15 (resorufin) and have emission wavelength (595 nm) that is easily
 16 distinguished from FAM emission. In accordance with the invention the
 17 chromophore is synthesized in such a way as to incorporate a linker structure
 18 for further functionalization to the desired phosphoramidite reagents. The
 19 preparation of preferred examples of these reagents **37** suitable for DNA
 20 synthesis, is shown in **Reaction Scheme 9**. Generally speaking in reagent **37**
 21 R_{10} and R_{11} independently are H, -OR₁₂, -NHR₁₃, halogen, -O(CH₂)_nCH₃, -
 22 (CH₂)_nCH₃, -NO₂, -SO₃, -C(=O)NH₂, -N[(CH₂)_nCH₃]₂, O-alkyl or O-alkanoyl
 23 where the alkyl or alkanoyl group has 1 to 10 carbons, or -CN where n= 0 to
 24 5; h=1 to 20; and R_{12} and R_{13} are blocking groups compatible with ODN
 25 synthesis. R_{14} in the scheme is H or DMTr.



REACTION SCHEME 9

As is shown in the example of **Scheme 9** in general terms and also for a specific example, reaction of nitrosoresorcinol derivative (commercially available or synthesized in accordance with the state-of-the-art) and of 4-(3-hydroxypropyl)benzene-1,3-diol (obtained in accordance with Forchiassin *et al. J. Heterocyc. Chem.* **20**, 1983, 493-494.) and MnO_2 yielded a resazurin derivative contaminated with some resorufin derivative. This mixture was treated with NH_4OH and Zn dust to yield resorufin derivative **35** (specifically **35a** where R_{10} is OH, and R_{11} is H) contaminated with 2,3,4-trihydro-2H-pyrano[3,2-b]phenoxazin-9-one as major impurity. The latter mixture was treated with DMTrCl and pyridine, and then with trimethylacetic acid anhydride. The product **36** was then subjected to purification by chromatography on silica gel to give the DMTr-protected derivative of **36a**

1 (where R_{10} is $-\text{OC}(=\text{O})\text{C}(\text{CH}_3)_3$, R_{11} is H and R_{14} is DMTr). The pure
 2 DMTr-derivative was treated with $\text{TFA}/\text{CH}_2\text{Cl}_2$ to yield a single product **36b**
 3 after silica gel chromatography. Treatment of **36** (where R_{10} is
 4 $-\text{OC}(=\text{O})\text{C}(\text{CH}_3)_3$, R_{11} and R_{14} are H) with 2-cyanoethyl
 5 diisopropylchlorophosphoramidite gave the desired phosphoramidite reagent
 6 **37** (specifically **37a**) that is utilized to introduce the fluorophore into a desired
 7 ODN.



REACTION SCHEME 10

10 PPT Phosphoramidite

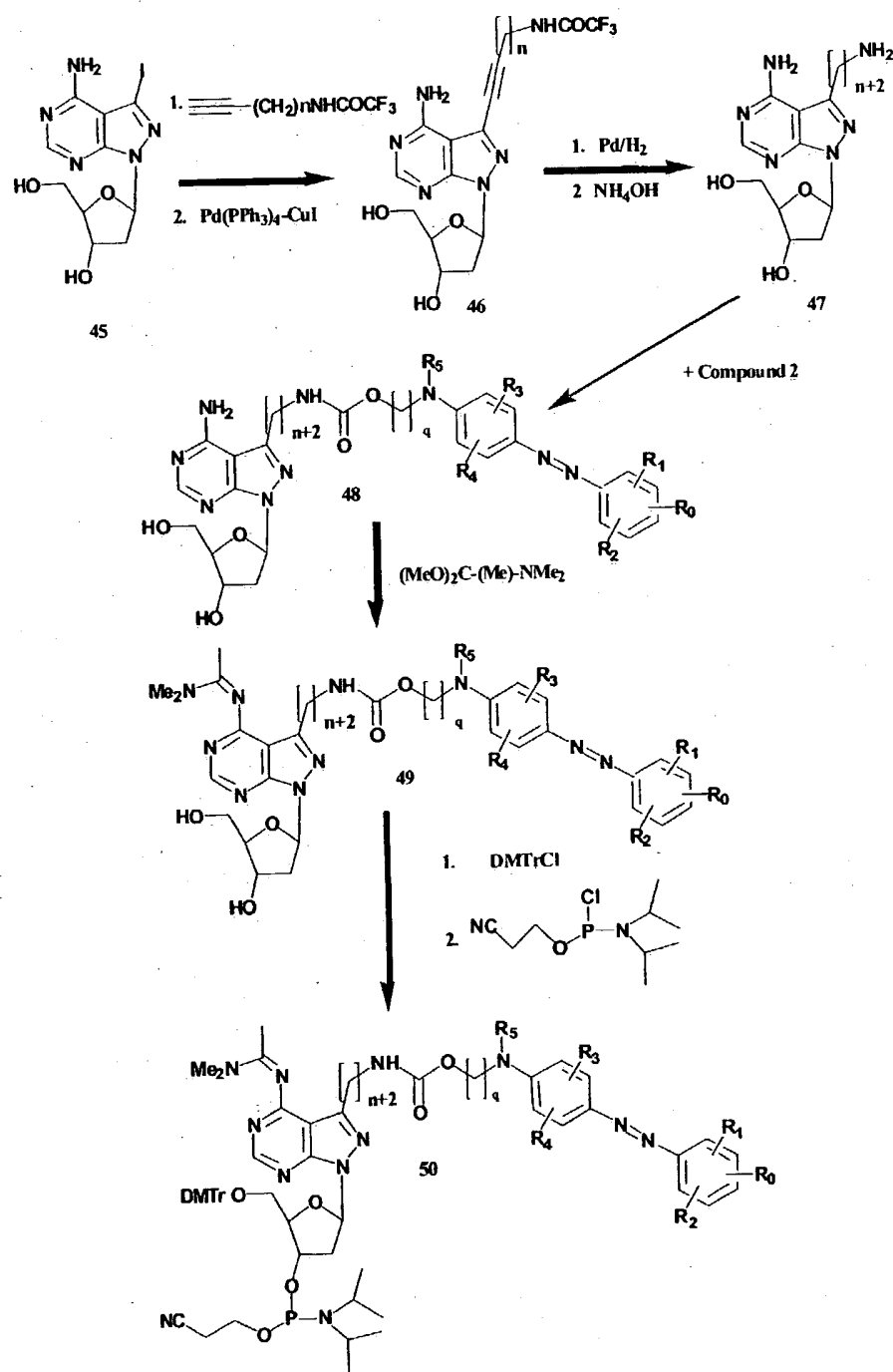
1 The synthesis of a phosphoramidite reagent incorporating a purple
2 fluorescent dye PPT **44** having excitation and emission wavelengths of 384
3 and 400 nm, respectively is shown in **Reaction Scheme 10** and in **Example**
4 **X**. In accordance with this scheme 6-chloro-3-n-butyluracil **38** and 2-(4-
5 aminophenyl)ethanol **39** are reacted to yield the phenyl substituted uracil
6 derivative **40**. The compounds **38** and **39** can be obtained in accordance
7 with the state-of-the-art and the chemical literature. Reaction of **40** with 5-
8 formyl-4,6-dichloro pyrimidine in DMF at room temperature affords the
9 tricyclic heterocycle **41**. Reduction of **41** in $\text{NH}_3/\text{Na}_2\text{S}_2\text{O}_4$ yields **42** which is
10 thereafter blocked as the toluoyl-derivative **43**. In the final step **43** is reacted
11 with 2-cyanoethyl diisopropylchlorophosphoramidite to yield the reagent
12 PPT cyanoethyl phosphoramidite **44** that is used to introduce the PPT
13 fluorophore into an ODN.

14 In still further embodiments, the fluorophores coumarin, resorufin and
15 PPT substituted with an alkylcarboxyl group serve as starting materials for the
16 synthesis of the corresponding phosphoramidite reagents. The fluorophores
17 coumarin, resorufin and PPT substituted with an alkylcarboxyl group are
18 either commercially available or can be synthesized in accordance with the
19 state-of-the-art. These compounds are activated on the alkylcarboxyl group
20 as the pentafluorophenyl esters. The activated esters are used to attach these
21 dyes to amine modified oligonucleotides.

22 Similarly, in still other embodiments, dUTP-labeled quenchers or
23 fluorophores are obtained for example in accordance with the teachings of
24 United States Patent No. 5,328,824). Furthermore, the phosphoramidite of 7-
25 labeled pyrazolo[3,4-d]pyrimidine-labeled quenchers or fluorophores are
26 synthesized according to the teaching of 5,824,796 (incorporated herein by
27 reference) and can be used for labeling of oligonucleotides.

28
29 **PPG Red Dye-based and Other Phosphoramidite Reagents for**
30 **Oligonucleotide Synthesis.**

1 In another embodiment the red dye 13 quencher is attached to the 3-
2 position of pyrazolo[5,4-d]pyrimidines (PP) or the 5-position of a pyrimidine.
3 Referring now to **Scheme 11** itself, the starting material is
4 5-(4-amino-3-iodopyrazolo[5,4-d]pyrimidinyl)-2-(hydroxymethyl)oxolan-3-ol
5 **45** which is available in accordance with the publication *Seela et al. J. Chem.*
6 *Soc., Perkin. Trans., 1 (1999, 479-488)* incorporated herein by reference.
7 Compound **45** is first reacted with N-propynyl-2,2,2-trifluoroacetate (or a
8 homolog thereof where in the scheme **n** is 1 to 10) and then with Pd(PPh₃)₄-
9 CuI to give the alkyne derivative **46**. Pd/H₂ reduction of **46** followed by
10 ammonium hydroxide treatment gives the aminoalkyl derivative **47** (PPA').
11 Reaction of PPA' with compound **2** (available as disclosed in connection
12 with **Reaction Scheme 1**) yielded substituted PPA'-Red 13 **48**. Reaction of
13 **48** with (1,1-dimethoxyethyl)dimethylamine blocks the amino group of the
14 pyrazolo[5,4-d]pyrimidine to yield **49**. **49** is first reacted with DMTrCl and
15 then with 2-cyanoethyl diisopropylchlorophosphoramidite to give the
16 DMTrCl blocked PPA'-Red 13 phosphoramidite **50**.



REACTION SCHEME 11

1 In still other embodiments starting with the deoxyriboside of 6-amino-
2 5-hydroxy-3-iodo-pyrazolo[5,4-d]pyrimidin-4-one (3-Iodo-PPG) the
3 phosphoramidite reagent containing the Red 13 dye covalently linked to the
4 3-Iodo-PPG moiety is synthesized with reactions analogous to those shown
5 in **Reaction Scheme 11**. Similarly starting with 5-aminopropyldeoxyuridine
6 the phosphoramidite reagent containing the Red 13 dye covalently linked to
7 5-aminopropyl-deoxyuridine is synthesized.

8 It will be clear to those skilled in the art in light of the foregoing
9 disclosure that the pyrazolopyrimidine-Red-13- or uridine-Red 13-based
10 phosphoramidites within the scope of this invention may contain various
11 linkers between the pyrazolopyrimidine and uracil bases and the Red 13
12 quenchers, to the full extent such linkers are available in accordance with the
13 state of the art and this disclosure.

14

15 EXAMPLES

16

17 The methods and compositions of the present invention can be used
18 with a variety of techniques, both currently in use and to be developed, in
19 which hybridization of an oligonucleotide to another nucleic acid is involved.
20 These include, but are not limited to, techniques in which hybridization of an
21 oligonucleotide to a target nucleic acid is the endpoint; techniques in which
22 hybridization of one or more oligonucleotides to a target nucleic acid
23 precedes one or more polymerase-mediated elongation steps which use the
24 oligonucleotide as a primer and the target nucleic acid as a template;
25 techniques in which hybridization of an oligonucleotide to a target nucleic
26 acid is used to block extension of another primer; techniques in which
27 hybridization of an oligonucleotide to a target nucleic acid is followed by
28 hydrolysis of the oligonucleotide to release an attached label; and techniques
29 in which two or more oligonucleotides are hybridized to a target nucleic acid
30 and interactions between the multiple oligonucleotides are measured. The

1 conditions for hybridization of oligonucleotides, and the factors which
2 influence the degree and specificity of hybridization, such as temperature,
3 ionic strength and solvent composition, are well-known to those of skill in the
4 art. See, for example, *Sambrook et al., supra*; *Ausubel et al., supra*; *Innis et*
5 *al. (eds.) PCR Protocols*, Academic Press, San Diego, 1990; *Hames et al.*
6 *(eds.) Nucleic Acid Hybridisation: A Practical Approach*, IRL Press, Oxford,
7 1985; and *van Ness et al. (1991) Nucleic Acids Res. 19:5143-5151*.

8 Hybridization Probes

9 In one application of the present invention, one or more FL-
10 oligonucleotide conjugates are used as probe(s) to identify a target nucleic
11 acid by assaying hybridization between the probe(s) and the target nucleic
12 acid. A probe may be labeled with any detectable label of the present
13 invention, or it may have the capacity to become labeled either before or after
14 hybridization, such as by containing a reactive group capable of association
15 with a label or by being capable of hybridizing to a secondary labeled probe,
16 either before or after hybridization to the target. As a basis of this technique it
17 is noted that conditions for hybridization of nucleic acid probes are well-
18 known to those of skill in the art. See, for example, *Sambrook et al.*,
19 *MOLECULAR CLONING: A LABORATORY MANUAL*, Second Edition,
20 Cold Spring Harbor Laboratory Press (1989); *Ausubel et al.*, *CURRENT*
21 *PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley & Sons (1987,
22 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996); *Hames et al. (eds.)*
23 *Nucleic Acid Hybridization: A Practical Approach*, IRL Press, Oxford, 1985;
24 and *van Ness et al. Nucleic Acids Res. 19:5143-5151(1991)*.

25 Hybridization can be assayed (*i.e.*, hybridized nucleic acids can be
26 identified) by distinguishing hybridized probe from free probe by one of
27 several methods that are well-known to those of skill in the art. These
28 include, but are not limited to, attachment of target nucleic acid to a solid
29 support, either directly or indirectly (by hybridization to a second, support-
30 bound probe or interaction between surface-bound and probe-conjugated

1 ligands) followed by direct or indirect hybridization with probe, and washing
2 to remove unhybridized probe; determination of nuclease resistance; buoyant
3 density determination; affinity methods specific for nucleic acid duplexes
4 (e.g., hydroxyapatite chromatography); interactions between multiple probes
5 hybridized to the same target nucleic acid; *and other known techniques*. See,
6 for example, *Falkow et al.*, United States Patent No. 4,358,535; *Urdea et al.*,
7 United States Patent Nos. 4,868,105 and 5,124,246; *Freifelder, Physical*
8 *Biochemistry*, Second Edition, Freeman & Co., San Francisco, 1982;
9 Sambrook, et al., *supra*; Ausubel et al., *supra*; and Hames et al., *supra*.

10

11 Assays Utilizing Labeled Probes, Hydrolyzable Probe and Labeled Primers

12

13 Additional applications for oligonucleotide conjugates containing a
14 fluorophore and quencher are found in assays in which a labeled probe is hybridized
15 to a target and/or an extension product of a target, and a change in the physical state
16 of the label is effected as a consequence of hybridization. A probe is a nucleic acid
17 molecule that is capable of hybridizing to a target sequence in a second nucleic acid
18 molecule. By way of example, one assay of this type, the hydrolyzable probe assay,
19 takes advantage of the fact that many polymerizing enzymes, such as DNA
20 polymerases, possess intrinsic 5'-3' exonucleolytic activities. Accordingly, if a
21 probe is hybridized to a sequence that can serve as a template for polymerization
22 (for instance, if a probe is hybridized to a region of DNA located between two
23 amplification primers, during the course of an amplification reaction), a
24 polymerizing enzyme that has initiated polymerization at an upstream amplification
25 primer is capable of exonucleolytically digesting the probe. Any label attached to
26 such a probe will be released, if the probe is hybridized to its target and if
27 amplification is occurring across the region to which the probe is hybridized.
28 Released label is separated from labeled probe and detected by methods well-known
29 to those of skill in the art, depending on the nature of the label. For example,
30 radioactively labeled fragments can be separated by thin-layer chromatography and
31 detected by autoradiography; while fluorescently-labeled fragments can be detected
32 by irradiation at the appropriate excitation wavelengths with observation at the

1 appropriate emission wavelengths. This basic technique is described for example in
2 United States Patent No. 5,210,015 incorporated herein by reference.

3 In a variation of this technique, a probe contains both a fluorescent label and
4 a quenching agent, which quenches the fluorescence emission of the fluorescent
5 label. In this case, the fluorescent label is not detectable until its spatial
6 relationship to the quenching agent has been altered, for example by
7 exonucleolytic release of the fluorescent label from the probe. Thus, prior to
8 hybridization to its target sequence, the dual fluorophore/quencher labeled
9 probe does not emit fluorescence. Subsequent to hybridization of the
10 fluorophore/quencher-labeled probe to its target, it becomes a substrate for the
11 exonucleolytic activity of a polymerizing enzyme which has initiated
12 polymerization at an upstream primer. Exonucleolytic degradation of the
13 probe releases the fluorescent label from the probe, and hence from the
14 vicinity of the quenching agent, allowing detection of a fluorescent signal
15 upon irradiation at the appropriate excitation wavelengths. This method has
16 the advantage that released label does not have to be separated from intact
17 probe. Multiplex approaches utilize multiple probes, each of which is
18 complementary to a different target sequence and carries a distinguishable
19 label, allowing the assay of several target sequences simultaneously.

20 The use of FL-ODN-Q-DPI₃ conjugates in this and related methods
21 allows greater speed, sensitivity and discriminatory power to be applied to
22 these assays. In particular, the enhanced ability of MGB-oligonucleotide
23 conjugates to allow discrimination between a perfect hybrid and a hybrid
24 containing a single-base mismatch facilitates the use of hydrolyzable probe
25 assays in the identification of single-nucleotide polymorphisms and the like,
26 as described in the publication WO 995162A2, incorporated herein by
27 reference. Examples 13 and 14 illustrate the utility of FL-ODN-Q- DPI₃
28 conjugates in this type of assay. It will be clear to those of skill in the art that
29 compositions and methods, such as those of the invention, that are capable of
30 discriminating single-nucleotide mismatches will also be capable of
31 discriminating between sequences that have multiple mismatches with respect

1 to one another.

2 Another application embodiment uses a self-probing primer with an
3 integral tail, where the quencher/fluorophore is present in the hairpin, that can
4 probe the extension product of the primer and after amplification hybridizes
5 to the amplicon in a form that fluoresces. The probing of a target sequence
6 can thereby be converted into a unimolecular event (Whitcombe, D. et al.,
7 Nat. Biotech., 17: 804-807 (1999)).

8

9 Fluorescence Energy Transfer

10 In further applications of the novel compositions of this invention,
11 oligonucleotide conjugates containing a fluorophore/quencher pair (FL-ODN-
12 Q) are used in various techniques which involve multiple fluorescent-labeled
13 probes. In some of these assays changes in properties of a fluorescent label
14 are used to monitor hybridization. For example, fluorescence resonance
15 energy transfer (FRET) has been used as an indicator of oligonucleotide
16 hybridization. In one embodiment of this technique, two probes are used,
17 each containing a fluorescent label and a quencher molecule respectively.
18 The fluorescent label is a donor, and the quencher is an acceptor, wherein the
19 emission wavelengths of the donor overlap the absorption wavelengths of the
20 acceptor. The sequences of the probes are selected so that they hybridize to
21 adjacent regions of a target nucleic acid, thereby bringing the fluorescence
22 donor and the acceptor into close proximity, if target is present. In the
23 presence of target nucleic acid, irradiation at wavelengths corresponding to
24 the absorption wavelengths of the fluorescence donor will result in emission
25 from the fluorescence acceptor. These types of assays have the advantage that
26 they are homogeneous assays, providing a positive signal without the
27 necessity of removing unreacted probe. For further details and additional
28 examples of these assays which are *per se* known in the art, see, for example,
29 European Patent Publication 070685; and the publication Cardullo et al.
30 (1988) *Proc. Natl. Acad. Sci. USA* 85: 8790-8794, both of which are

1 incorporated herein by reference. Additional applications of the novel
2 compositions of the present invention are in those and related techniques in
3 which interactions between two different oligonucleotides that are both
4 hybridized to the same target nucleic acid are, measured. The selection of
5 appropriate fluorescence donor/fluorescence acceptor pairs will be apparent to
6 one of skill in the art, based on the principle that, for a given pair, the
7 emission wavelengths of the fluorescence donor will overlap the absorption
8 wavelengths of the acceptor. The enhanced ability of DPI₃-oligonucleotide
9 conjugates to distinguish perfect hybrids from hybrids containing a single
10 base mismatch facilitates the use of FRET-based techniques in the
11 identification of single-nucleotide polymorphisms and the like.

12 In another application of the novel compositions of the invention, the
13 fluorescence of the FL-ODN-Q conjugate is quenched in its native state. But,
14 after hybridization with the intended target the spatial arrangement of the
15 fluorophore and quencher moieties are changed such that fluorescence occurs.
16 For this basic technique see for example *Tyagi et al.*, Nat. Biotech., 16: 49-53
17 (1998); and United States Patent No. 5,876,930, both of which are
18 incorporated herein by reference.

19 It should be understood that in addition to the fluorophores which are
20 found in accordance with the present invention especially useful to be used
21 with the quenchers of the invention, and which fluorophores are incorporated
22 into ODNs in accordance with the invention, a person of ordinary skill may
23 choose additional fluorophores to be used in combination with the quenchers
24 of the present invention, based on the optical properties described in the
25 literature, such as the references: *Haugland Handbook of Fluorescent Probes*
26 and Research Chemicals, Six Edition, Eugene, OR. pp. 235-236. 1996;
27 *Berlman*, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd,
28 Accademic Press, New York, 1971; Du et al., PhotochemCAD. A
29 Computer-Aided Design and Research Tool in Photochemistry, Photochem.
30 Photobiol. 68, 141-142 (1998). Therefore the use of the novel ODN quencher

1 conjugates in combination with these known fluorophores is considered
2 within the scope of the invention.

3 In another application, the minor groove binder, DPI₃, is coupled to a
4 quencher in a FL-ODN-Q- CDPI₃ conjugate to improve signal to noise ratios
5 (See Table 2). Preferred quenchers are the quenchers of Formula 6 and more
6 preferred the quenchers are 8-11, 12-16 and 30.

7 Additional quenchers suitable for use in combination with the novel
8 fluorophores (34, 37 and 44) of the invention include dabcylnitrothiazole,
9 TAMRA, 6-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino) hexanoic acid , 6-
10 carboxy-X-rhodamine (Rox) and QSY-7 .

11 Another application of the novel fluorophore/quencher pairs of the
12 invention is to incorporate the pair into enzyme substrates, where
13 fluorescence is quenched because of the proximity of the fluorophore and
14 quencher. However, after an enzyme cleaves the substrate the fluorophore
15 and quencher become separated and fluorescence is observed., An example of
16 this technique is described below using the phosphodiesterase enzyme. It will
17 be clear to those schooled in the art that suitable substrates containing both
18 the novel quenchers and fluorophores can be constructed for enzymes that
19 cleave substrates.

20

21 Oligonucleotide Arrays

22 In another application, FL-ODNs of the present invention are utilized
23 in procedures employing arrays of oligonucleotides. Examples for this
24 technique that is *per se* known in the art include sequencing by hybridization
25 and array-based analysis of gene expression. In these procedures, an ordered
26 array of oligonucleotides of different known sequences is used as a platform
27 for hybridization to one or more test polynucleotides, nucleic acids or nucleic
28 acid populations. Determination of the oligonucleotides which are hybridized
29 and alignment of their known sequences allows reconstruction of the
30 sequence of the test polynucleotide. For a description of these techniques see

1 for example, United States Patent Nos. 5,492,806; 5,525,464; 5,556,752;
2 and PCT Publications WO 92/10588 and WO 96/17957 all of which are
3 incorporated herein by reference. Materials for construction of arrays include,
4 but are not limited to, nitrocellulose, glass, silicon wafers and optical fibers.

5 6 STRUCTURAL CONSIDERATIONS

7
8 The terms oligonucleotide, polynucleotide and nucleic acid are used
9 interchangeably to refer to single- or double-stranded polymers of DNA or
10 RNA (or both) including polymers containing modified or non-naturally-
11 occurring nucleotides, or to any other type of polymer capable of stable base-
12 pairing to DNA or RNA including, but not limited to, peptide nucleic acids
13 which are disclosed by *Nielsen et al.* (1991) *Science* **254**:1497-1500; bicyclo
14 DNA oligomers (*Bolli et al.* (1996) *Nucleic Acids Res.* **24**:4660-4667) and
15 related structures. One or more MGB moieties and/or one or more
16 fluorescent labels, and quenching agents can be attached at the 5' end, the 3'
17 end or in an internal portion of the oligomer. A preferred MGB in accordance
18 with the invention is DPI₃ and the preferred quencher is red 13 amide.

19 Preferred in the present invention are DNA oligonucleotides that are
20 single-stranded and have a length of 100 nucleotides or less, more preferably
21 50 nucleotides or less, still more preferably 30 nucleotides or less and most
22 preferably 20 nucleotides or less with a lower limit being approximately 5
23 nucleotides.

24 Oligonucleotide conjugates containing a fluorophore/quencher pair
25 with or without an MGB may also comprise one or more modified bases, in
26 addition to the naturally-occurring bases adenine, cytosine, guanine, thymine
27 and uracil. Modified bases are considered to be those that differ from the
28 naturally-occurring bases by addition or deletion of one or more functional
29 groups, differences in the heterocyclic ring structure (*i.e.*, substitution of
30 carbon for a heteroatom, or *vice versa*), and/or attachment of one or more

1 linker arm structures to the base. The modified nucleotides which may be
2 included in the ODN conjugates of the invention include 7-deazapurines and
3 their derivatives and pyrazolopyrimidines (described in PCT WO 90/14353
4 incorporated herein by reference); and in co-owned and co-pending
5 application serial number 09/054,630.

6 Preferred base analogues of this type include the guanine analogue
7 6-amino-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (ppG or PPG) and the
8 adenine analogue 4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine (ppA or PPA).
9 Also of use is the xanthine analogue 1*H*-pyrazolo[5,4-*d*]pyrimidin-4(5*H*)-
10 6(7*H*)-dione (ppX). These base analogues, when present in an
11 oligonucleotide, strengthen hybridization and improve mismatch
12 discrimination. All tautomeric forms of naturally-occurring bases, modified
13 bases and base analogues may be included in the oligonucleotide conjugates
14 of the invention.

15 Similarly, modified sugars or sugar analogues can be present in one or
16 more of the nucleotide subunits of an oligonucleotide conjugate in accordance
17 with the invention. Sugar modifications include, but are not limited to,
18 attachment of substituents to the 2', 3' and/or 4' carbon atom of the sugar,
19 different epimeric forms of the sugar, differences in the α - or β - configuration
20 of the glycosidic bond, and other anomeric changes. Sugar moieties include,
21 but are not limited to, pentose, deoxypentose, hexose, deoxyhexose, ribose,
22 deoxyribose, glucose, arabinose, pentofuranose, xylose, lyxose, and
23 cyclopentyl.

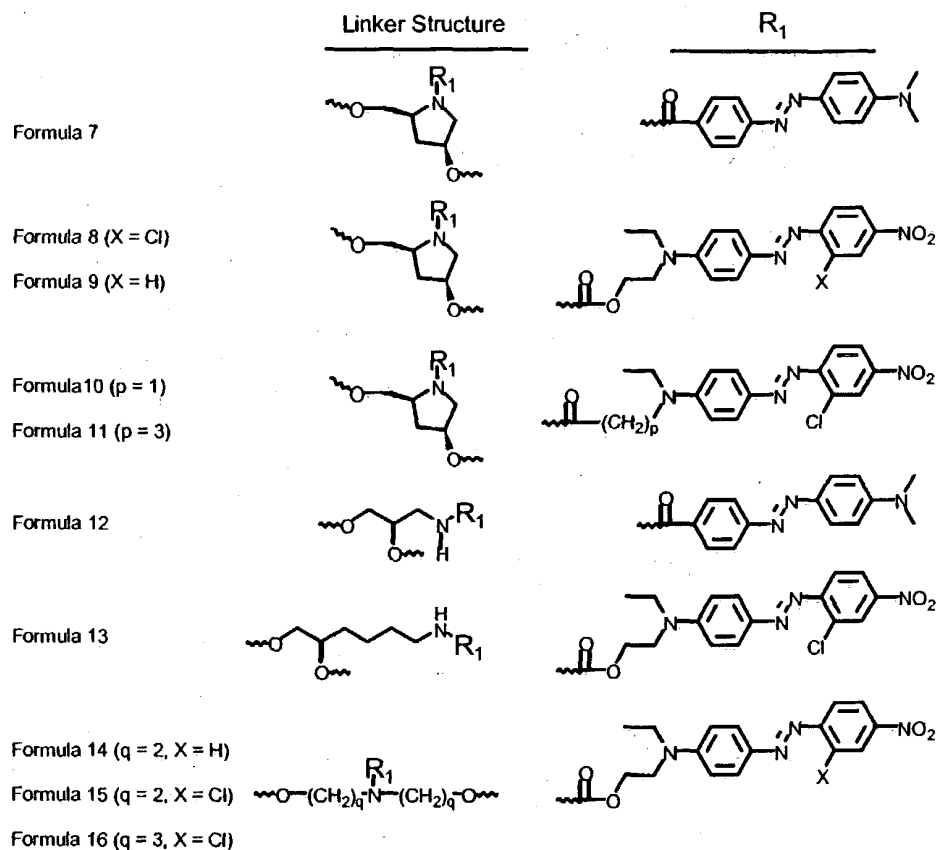
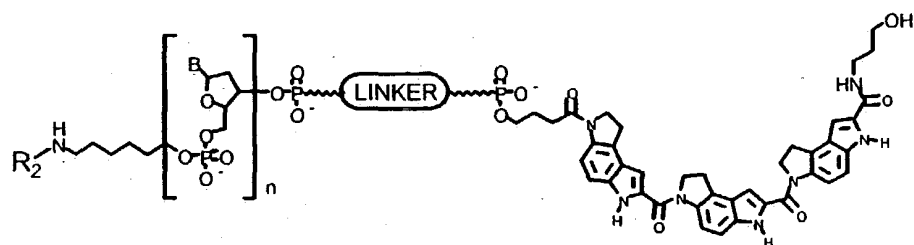
24 Modified internucleotide linkages can also be present in
25 oligonucleotide conjugates of the invention. Such modified linkages include,
26 but are not limited to, peptide, phosphate, phosphodiester, phosphotriester,
27 alkylphosphate, alkanephosphonate, thiophosphate, phosphorothioate,
28 phosphorodithioate, methylphosphonate, phosphoramidate, substituted
29 phosphoramidate and the like. Several further modifications of bases, sugars
30 and/or internucleotide linkages, that are compatible with their use in

1 oligonucleotides serving as probes and/or primers, will be apparent to those of
2 skill in the art.

3 Certain preferred embodiments of the invention involve the synthesis
4 of numerous phosphoramidites with various quencher chromophores and
5 linkers and their incorporation at the 3'-end of fluorogenic MGB ODNs as
6 shown in **Reaction Scheme 3**. Different fluorescent reporter groups (shown
7 in **Reaction Scheme 7**) were also incorporated into the oligonucleotide
8 probes and are described in the EXPERIMENTAL section. The fluorogenic
9 properties of these ODN conjugates are described in **Table 2**. In other
10 embodiments MGB molecules, due to their desirable improved hybridization
11 properties, were incorporated into oligonucleotides containing both a
12 fluorophore and a quencher, without loss in hybridization specificity,
13 fluorescent quenching and fluorescent signal. The flat aromatic quencher
14 residue coupled to the neighboring aromatic DPI₃ residue, have strict
15 geometric requirements since the linker between the oligonucleotide and the
16 DPI₃ residue must be flexible enough to allow positioning of the DPI₃ in the
17 minor groove after DNA duplex formation.

18 Characteristics of Reagents of the Invention

19 A number of FL-ODN-Q-DPI₃ conjugates synthesized with the
20 reagents and methods of the invention are shown in **Formulas 7 to 16**, where
21 *n* specifies the number of bases in the oligonucleotide and R₂ is either FAM or
22 TAMRA. "B" signifies a heterocyclic base attached to the deoxyribose sugar
23 moiety.



- 1 Formulas 7 to 16
- 2 The quenchers incorporated in the compounds represented by
- 3 **Formulas 7-16** are the commercially available 2-[4-(4-nitrophenylazo)-N-
- 4 ethylphenylamino]ethanol (Disperse Red 1), 2-[4-(2-chloro-4-
- 5 nitrophenylazo)-N-ethylphenylamino]ethanol (Disperse Red 13) and 2-[4-
- 6 (dimethylamino)phenylazo]benzoic acid, identified in this invention as Red1,
- 7 Red 13 and dabcy1 respectively.
- 8
- 9 UV Properties of Red 13 and Dabcy1 Oligonucleotide Conjugates

1 **Figure 2** shows the absorbance properties of the red13 chromophore
2 (**Formula 8**, without DPI₃) in comparison to dabcyl (**Formula 7**, without
3 DPI₃ when incorporated at the 3'-end of an otherwise unmodified DNA
4 probe. The broader absorbance (especially at long wavelengths) of the red13
5 chromophore is a clear advantage. Note that the λ_{max} for red13 is at 522 nm
6 whereas the λ_{max} for dabcyl is 479 nm. The absorbance of red13 is ideal for
7 quenching of fluorescein (emission max = 525 nm) but also overlaps with the
8 fluorescence emission of other common laser dyes.

9

10 **Quenching Properties of DPI₃ Probes with Various Quenchers and Linkers.**

11 For the 10 fluorogenic probes described in **Formulas 7 to 16** the
12 fluorescence of a standard solution of each probe was measured before and
13 after digestion with snake venom phosphodiesterase (PDE), as described in
14 the EXPERIMENTAL section. This PDE assay allows the quenching
15 properties of each probe to be compared. Fluorescence of the digested probe
16 (signal) divided by the initial fluorescence (noise) gave a signal to noise ratio
17 (S/N), presented in **Table 1**. Larger numbers for S/N reflect more efficient
18 fluorescent quenching (lower fluorescent background) of the intact probe.

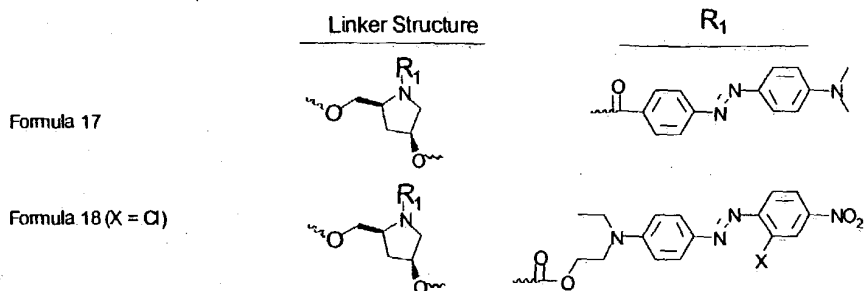
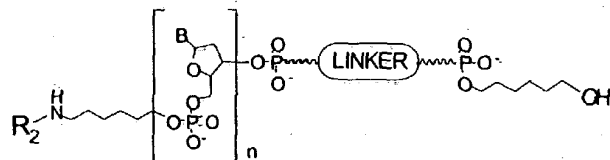
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1 **Table 1. Effect of different quenchers and linkers on fluorogenic probes**
2 **shown in Formulas 7-16.**

Formula # (quencher)	S/N ^a (R ₂ = FAM)	S/N ^a (R ₂ = TAMRA)
7 (dabcyl)	16	13
8 (red13)	21	21
9 (red1)	24	21
10 (red13)	13	33
11 (red13)	27	21
12 (dabcyl)	13	7
13 (red13)	23	21
14 (red1)	19	3
15 (red13)	24	4
16 (red13)	22	24

3
4 ^aSignal to noise (S/N) was determined using the phosphodiesterase assay
5 described in Example 13. The ODN sequence was 5'-gagggatgtaaaat
6 (SEQUENCE Id. No. 1). The fluorophores (R₂) studied here is either 6-
7 carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (TAMRA).

8
9 It is clear from the data in **Table 1** that the red13 chromophore and the
10 closely related red1 chromophore are better quenchers for both FAM and
11 TAMRA with a variety of linkers than dabcyl. The linker can affect
12 quenching by the red13 chromophore. For example, **Formula 14** and
13 **Formula 15** worked well with FAM, but had poor quenching efficiency for
14 TAMRA. It is somewhat surprising that dabcyl worked so well, especially for
15 the TAMRA probes. As described below, effective FRET quenching by
16 dabcyl is a specific case for MGB probes.



Formulas 17 to 18

Comparison of Quenching Properties of DPI₃ Probes and Probes without DPI₃.

To further show the advantages of the red13 quencher chromophore, fluorogenic probes with a 3'-hexanol blocking group (without MGB) were compared. The structure and fluorescent properties of 13 fluorogenic probes with the same sequence were compared using the PDE assay. A red-sensitive detector was used in this study (Table 2) whereas a blue-sensitive detector was used in the study shown in Table 1 (S/N for identical ODNs are different because different detectors have different sensitivities for the same fluorophore). The following structural variables are summarized in Table 2: Probe type (no-MGB vs. MGB), Quencher (dabcyl vs. Red13 vs. Red13 amide), and Reporter dye (FAM vs. TAMRA).

Table 2. Fluorescent properties of oligonucleotides with various Quenchers / Fluorophores¹.

<u>Formula #</u>	<u>Probe type</u>	<u>Quencher</u>	<u>FAM (S/N)</u>	<u>TAMRA (S/N)</u>
17	no- DPI ₃	dabcyl	4.7	3.9
18	no- DPI ₃	red13	11.6	5.8
7	DPI ₃	dabcyl	23	23.5
8	DPI ₃	red13	35	108
30 (RI=2- Cl, t=ν=3)	DPI ₃	red13 amide	48	97

3 ¹Signal to noise (S/N) was determined using the phosphodiesterase assay
4 described above. The ODN sequence was 5'-gagggatgtaaaaat (**SEQUENCE**
5 **Id. No. 1**). The linker structure of the dabcyI or red13 quenchers (**Q**) is
6 shown in **Formulas 7 and 8** respectively. The linker structure of the red13
7 amide is shown in **30 Reaction Scheme 7**, R_0 is NO_2 , $R_1=2\text{-Cl}$, $R_2=R_3=R_4=$
8 H ; $t=v=3$.

10 It is clear from the data shown in **Table 2**, that in the probes which do
11 not contain DPI₃ the dye Red 13 quencher works better than dabcyI for both
12 FAM and TAMRA. In DPI₃ containing probes, the dye Red13 works better in
13 combination with FAM and much better in combination with TAMRA. Both
14 **8** and **30** work better in DPI₃ containing probes with both fluorophores, with
15 **30** showing the best S/N ratio for FAM. Thus, it was found that the Red13
16 chromophore is a more efficient quencher than dabcyI for long wavelength
17 fluorescent reporter groups. For the most commonly used fluorophore (FAM)
18 a 2.5-fold increase in S/N was observed for standard (no-DPI₃) probes. This
19 improved quenching by red13 is consistent with the increased spectral overlap
20 presented in **Figure 2** and a standard FRET mechanism. The increased S/N
21 of both **8** and **30** when incorporated into the DPI₃ probes is dramatic and

1 surprising. The combination of the red13 quencher and the DPI₃ resulted in a
2 10-fold increase in S/N for FAM quenching and a 28-fold increase in S/N for
3 TAMRA quenching.

4 It is surprising and that the DPI₃ residue helps improve fluorescent
5 quenching by the dabcyI and red13 chromophores. Without wishing to be
6 bound by theory, it is presently postulated that the random coil conformation
7 of the fluorogenic probe in solution is more structured in the DPI₃ probes such
8 that the average distance between the fluorophore and quencher is closer than
9 in probes without MGB. This closer average distance in the DPI₃ probes
10 (tighter coil) would give rise to more efficient FRET. The exact nature of this
11 interaction is not known, but UV spectra of the quencher and dye
12 chromophores are not affected by the DPI₃. This is in contrast to the
13 fluorogenic hairpin probes where the UV spectra are changed by the
14 constrained conformation (collisional quenching).

15 Performance of Fluorogenic DPI₃ Probes in a "Real-Time" PCR Assay.

16 DPI₃ probes prepared with 5'-fluorescein and the red13 amide linker
17 were tested in the 5'-nuclease assay to see if the hybridization properties were
18 compatible with the linker system. As shown in **Figure 3**, both dabcyI and
19 Red13 worked as quenchers for fluorescein in the 5'-nuclease assay when
20 used in MGB probes. Red13 performed better than dabcyI as evidenced by
21 the lower initial fluorescence (background) and the higher plateau after PCR.
22 Current commercially available thermal-cycling fluorimeters can not read
23 longer wavelength dyes in real-time PCR, but the red13 chromophore was
24 shown to give a good S/N with TAMRA containing probes in an end point
25 analysis after PCR.

26 According to another general method, the 5'-fluorophore-ODN-Q-
27 MGB conjugates of the instant invention have improved performance in
28 assays designed to detect DNA targets by direct hybridization. A basic
29 description of this method is found in United States Patent No. 5,876,930,
30 incorporated herein by reference. In this assay format, the non-hybridized

1 probes (quenched by FRET) become fluorescent upon forming a rigid duplex
2 structure, thereby separating the quencher and fluorophore.

3 Red13 Chromophore Quenches a Broad Range of Fluorescent Reporter 4 Groups

5 A series of DPI₃ probes with the red13 amide were prepared with
6 several different fluorescent reporter groups to examine the effective range of
7 quenching. Probes were digested with PDE as usual and showed good S/N
8 for dyes which emit from 458 -665 nm.

9 **Table 3. Performance of Fluorogenic DPI₃ Probes with Various**
10 **Fluorophores.**

<u>Fluorophore (FL)</u>	<u>Ex 8 (nm)</u>	<u>Em 8 (nm)</u>	<u>S/N</u>
coumarin	378	458	32
FAM	488	522	63
Cy3	541	565	61
TAMRA	547	582	37
resorufin	549	595	110
Cy5	641	665	36

11
12 *The structure of the fluorogenic probes was FL-ODN-Q-CDPI₃ where Q is the*
13 *red13 amide and the ODN sequence was 5'- GTC CTG ATT TTA C*
14 *(SEQUENCE Id. No. 2). The fluorophores FAM, TAMRA, cy3 and cy5 were*
15 *incorporated using commercially available phosphoramidite reagents. The*
16 *coumarin and resorufin fluororophores were incorporated using*
17 *phosphoramidites 34 and 37 which were prepared as described below.*

18 The fluorescent emission is well separated from FAM, as shown in the
19 overlaid spectra in **Figure 4**. As shown in **Table 3**, the resorufin
20 fluorescence is also quenched by the red13 chromophore. Thus the resorufin
21 phosphoramidite has excellent properties for use in FRET probes and in
22 combination with FAM for multicolor analysis.

23 As shown in **Table 3**, the coumarin fluorescence is also quenched by

1 the red13 chromophore. Thus, the coumarin phosphoramidite reagent can be
2 incorporated in FRET probes and particularly in combination with FAM for
3 multicolor analysis.

4 5 FRET-based enzyme substrates

6 The improved quencher molecules can be used in other FRET based
7 assay systems. According to another general application of the invention, a
8 quencher molecule and fluorophore are attached to an enzyme substrate,
9 which through its catalytic action on this Q-substrate-fluorophore conjugates
10 cleaves and separates the Q and fluorophore molecules. For example, the
11 pentafluorophenyl activated ester **11** shown in **Reaction Scheme 3** can be
12 used for labeling lysine residues of peptides for studying proteolytic enzymes.

13 14 EXPERIMENTAL PROCEDURES

15 16 General Experimental

17 All air and water sensitive reactions were carried out under a slight
18 positive pressure of argon. Anhydrous solvents were obtained from Aldrich
19 (Milwaukee, WI). Flash chromatography was performed on 230-400 mesh
20 silica gel. Melting points were determined on a Mel-Temp melting point,
21 apparatus in open capillary and are uncorrected. Elemental analysis was
22 performed by Quantitative Technologies Inc. (Boundbrook, NJ). UV-visible
23 absorption spectra were recorded in the 200-400-nm range on a UV-2100
24 (Shimadzu) or a Lambda 2 (Perkin Elmer) spectrophotometers. ¹H NMR
25 spectra were run at 20EC on a Bruker WP-200 or on a Varian XL-200
26 spectrophotometer; chemical shifts are reported in ppm downfield from
27 Me₄Si. Thin-layer chromatography was run on silica gel 60 F-254 (EM
28 Reagents) aluminum-backed plates.

29 30 Example 1

1 2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl
 2 (2S,4R)-2-{{bis(4-methoxyphenyl)phenylmethoxy)methyl}-4-
 3 {[bis(methylethyl)amino](2-cyano-
 4 ethoxy)phosphinoxy}pyrrolidinecarboxylate (5).

5
 6 2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (5S,3R)-3-
 7 hydroxy-5-(hydroxymethyl)pyrrolidinecarboxylate (3).

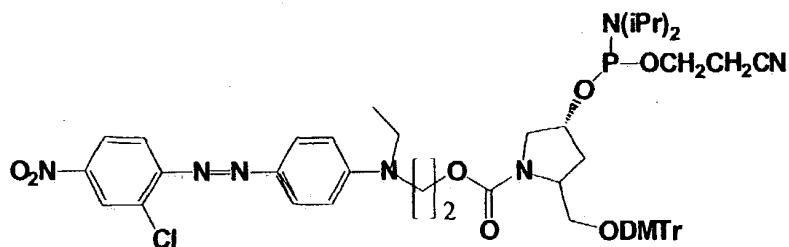
8 A solution of 2-[4-(2-chloro-4-nitrophenylazo)- *N*-
 9 ethylphenylamino]ethanol (Disperse Red 13, Aldrich Chemical Co., 9.0 g,
 10 25.80 mmol) and 4-nitrophenyl chloroformate (Aldrich Chemical Co., 9.4 g,
 11 46.61 mmol) in 90 ml of anhydrous pyridine was stirred at 70 °C for 40 min,
 12 affording intermediate 2. Ethanol (5.0 ml) was added to the reaction solution
 13 followed by trans-hydroxyprolinol (Reed et al. Bioorg. Chem. 2: 217-225
 14 (1991) (42 ml of a 0.5 M solution in ethanol) and triethylamine (3.2 ml). The
 15 resultant solution was stirred for 30 min at 70 °C. The solution was
 16 evaporated to dryness and the residue was suspended in 1 liter of water and
 17 extracted with ethyl acetate (3 x 500 ml). The pooled extracts were dried over
 18 sodium sulfate, filtered and evaporated. The residue was purified by silica gel
 19 chromatography eluting with a gradient of 0-10% methanol in ethyl acetate.
 20 The pure product fractions were evaporated and precipitated from ethyl
 21 acetate - ether: 9.2 g (59%); TLC (ethyl acetate), R_f = 0.25. ¹H NMR
 22 (DMSO-*d*₆) δ 8.43 (1H, d, *J* = 2.5 Hz), 8.25 (1H, dd, *J* = 9.0 and 2.4 Hz), 7.86
 23 (2H, d, *J* = 9.1 Hz), 7.78 (1H, d, *J* = 9.0 Hz), 6.96 (2H, d, *J* = 9.3 Hz), 4.88
 24 (1H, m), 4.67 (1H, t, *J* = 5.7 Hz), 4.19 (3H, m), 3.80 (1H, m), 3.73 (2H, t, *J* =
 25 5.4 Hz), 3.56 (2H, q), 3.46 (1H, t, *J* = 4.7 Hz), 3.27 (1H, m), 1.94 (1H, m),
 26 1.79 (1H, m), 1.17 (3H, t, *J* = 6.8 Hz). Anal. Calcd for C₂₂H₂₆ClN₅O₆ + 0.2
 27 H₂O: C, 53.32; H, 5.37; N, 14.13. Found: C, 53.24; H, 5.25; N, 13.99.

28
 29 2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (5S,3R)-5-
 30 {[bis(4-methoxyphenyl)phenylmethoxy)methyl]-3-

1 *hydroxypyrrolidinecarboxylate (4).*

2 To a solution of **3** (9.1 g, 18.53 mmol) in 130 ml of anhydrous
3 pyridine was added 6.26 g of dimethoxytrityl chloride. The solution was
4 stirred for 3 h. at room temperature and then poured into 300 ml of 5%
5 sodium bicarbonate solution. The mixture was extracted with ethyl acetate (2
6 x 300 ml) and the combined extracts were dried over sodium sulfate, filtered
7 and evaporated. The residue was purified by silica gel chromatography
8 eluting with a gradient of 20-0% hexane in ethyl acetate followed by a
9 gradient of 0-2% methanol in ethyl acetate. The chromatography eluent also
10 contained 1% triethylamine. The pure product fractions were combined
11 affording an amorphous solid: 12.66 g (86%); TLC (ethyl acetate), R_f = 0.44.
12 ^1H NMR (DMSO- d_6) δ 8.45 (1H, s), 8.26 (1H, d, J = 8.9 Hz), 7.82 (3H, m),
13 7.27 (4H, m), 7.16 (5H, m), 6.95-6.79 (6H, m), 4.95 (1H, m), 4.32 (1H, m),
14 4.14 (1H, m), 3.99 (2H, m), 3.73 (1H, m), 3.69 (6H, s), 3.56 (1H, m), 3.40-
15 3.30 (2H, m), 3.14 (1H, m), 2.10-1.82 (2H, m), 1.16 (3H, m), 1.06 (3H, t, J =
16 6.5 Hz). Anal. Calcd for $\text{C}_{43}\text{H}_{44}\text{ClN}_5\text{O}_8 + 0.2 \text{H}_2\text{O}$: C, 64.73; H, 5.61; N,
17 8.78. Found: C, 65.08; H, 5.70; N, 8.31.

18
19 2-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl}ethylamino)ethyl (2*S*,4*R*)-2-
20 {[bis(4-methoxyphenyl)phenylmethoxy]methyl}-4-{{bis(methylethyl)amino}(2-
21 cyano-ethoxy)phosphinoxy}pyrrolidinecarboxylate (**5**).



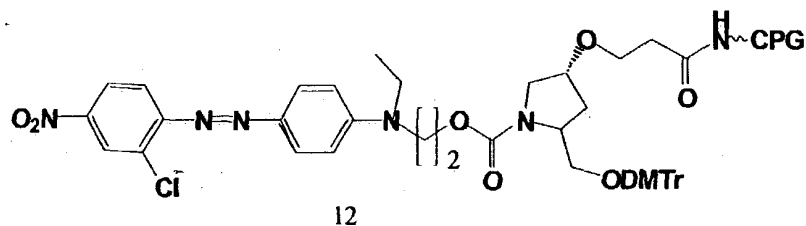
22
23 To a solution of **4** (12.63 g, 15.91 mmol) dissolved in 440 ml of
24 anhydrous methylene chloride, containing 8.0 ml of N,N-
25 diisopropylethylamine, was added 5.94 ml of 2-cyanoethyl

1 diisopropylchlorophosphoramidite. The solution was stirred 30 min under
 2 argon at room temperature. The reaction mixture was treated with 10 ml of
 3 methanol and poured into 400 ml of 5% sodium bicarbonate solution. The
 4 organic phase was dried over sodium sulfate and evaporated. The residue was
 5 purified by silica gel chromatography eluting with a gradient of 40-20%
 6 hexane in ethyl acetate (2% triethylamine). The pure product fractions were
 7 evaporated affording an amorphous solid: 14.75 g (93% yield). ^{31}P NMR
 8 ($\text{DMSO}-d_6$) δ 146.93 (singlet). Anal. Calcd for $\text{C}_{52}\text{H}_{61}\text{ClN}_7\text{O}_9 + 1.0 \text{ H}_2\text{O}$: C,
 9 61.68; H, 6.27; N, 9.68. Found: C, 61.44; H, 6.47; N, 9.35.

11 Example 2

12 RED 13-pyrrolidine-DMTr-CPG 12

14 *Synthesis of pentafluorophenyl ester (11) and RED 13_pyrrolidine_DMTr_CPG*
 15 *(12) Reactions Scheme 3.*



17 The pentafluorophenyl ester (11) is synthesized by the same method
 18 used for the synthesis of Compound 22 as described in Example 4 and
 19 Reaction Scheme 5.

21 RED 13-pyrrolidine-DMTr-CPG (12)

22 10 g of LCAA_CPG was combined with 5 ml of a 0.3 M solution of 11
 23 in DMF and agitated gently overnight, when it was filtered and washed with
 24 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether.
 25 Traces of ether were removed in vacuo (oil pump). Unreacted amino groups
 26 were acetylated by treating the CPG with 40 mL of dry pyridine and 5 mL of
 27 acetic anhydride. After swirling for 1.5 h, the CPG was filtered and washed

1 with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether.
2 Traces of ether were removed in vacuo (oil pump). The CPG was analyzed
3 for MMT loading by treating 3_5 mg of CPG in 25 mL of 1:1 / 70%
4 perchloric acid:methanol. The absorbance of the released MMT cation was
5 recorded at 472 nm and loading level was adjusted to be between 30_40
6 mmol / g of CPG using the equation:
7 $\text{MMT loading (mmol / g)} = A_{472} \times \text{volume (in mL)} \times 14.3$, wt of CPG (mg)

8 9 **Example 3**

10 **2-(4-Nitrophenyl)ethyl 3-(pyrrolo[4,5-e]indolin-7-ylcarbonyl)pyrrolo[4,5-**
11 **e]indoline-7-carboxylate (17). (Reaction Scheme 4)**

12
13 *2-(4-Nitrophenyl)ethyl 3-[(tert-butyl)oxycarbonyl]pyrrolo[4,5-e]indoline-7-*
14 *carboxylate (14).*

15 Ten grams (33.1 mmol) of 3-[(tert-butyl)oxycarbonyl]pyrrolo[3,2-
16 e]indoline-7-carboxylic acid (Boger, D.L., Coleman, R.S., Invergo, B.J.
17 (1987) *J. Org. Chem.* 52, 1521.), well dried, are placed into an argon filled
18 flask, and 84 mL of THF and 10.4 mL (66.2 mmol) of diethyl
19 azodicarboxylate (DEAD) are added. Then a dropping funnel is placed atop
20 the flask (flushed with argon) and a water bath (to cool the flask) is placed
21 under it. A solution of 17.3 g (66 mmol) of triphenylphosphine and 6.64 g
22 (39.7 mmol) of 2-(p-nitrophenyl) ethanol in 160 mL of ethyl ether is made.
23 This solution is added to the dropping funnel, and then to the reaction flask,
24 dropwise, with stirring. The reaction is allowed to proceed for an hour, at
25 which time, a TLC analysis is done (2:1 hexanes/ethyl acetate) examined by
26 UV (254 nm) to determine whether the reaction is complete. If it is complete,
27 then the baseline spot (bluish) will disappear and the product, with an R_f of
28 0.55, will appear as a dark spot. Often, especially if the reactants are not
29 entirely dry, another portion of triphenylphosphine and DEAD are required.
30 If so, a tenth of the original amounts is usually sufficient, i.e., 1.73 g of

1 triphenylphosphine and 1.04 mL of DEAD. These can be added neat to the
2 stirred solution. Allow to react another hour, after which another TLC
3 analysis usually reveals complete reaction. The product usually precipitates
4 out in part; this is collected by filtration and washed with methanol, then
5 recrystallized by dissolving in a minimum amount (typically, 80 - 100 mL) of
6 warm acetone and adding four times that volume of warm methanol. Cool to
7 4°C for several hours or perhaps overnight. The supernatant from the original
8 precipitation is saved and evaporated to a syrup or until dry. It too is
9 dissolved in a minimum amount of warm acetone; typically about 100 - 120
10 mL of warm acetone. The total amount of acetone used for the two
11 recrystallizations is usually approximately 200 mL. As before, an amount of
12 warm methanol equal to four times the amount of acetone is added. The
13 solution is cooled; crystallization begins almost at once but is allowed to
14 continue several hours to overnight. The recrystallizations are quite efficient,
15 but the product from the reaction supernatant is usually not quite as pure and
16 is purified by recrystallization. The yield is approximately 85%. (mp 191-
17 193°C) ¹H NMR (DMSO-*d*₆) δ 11.83 (s, 1H), 8.18 (d, J=8.5 Hz, 2H), 7.84
18 (br s, 1H), 7.64 (d, J=8.5 Hz, 2H), 7.25 (d, J=8.8 Hz), 6.96 (s, 1H), 4.56 (t,
19 J=6 Hz, 2H), 4.00 (t, J=8.8 Hz, 2H), 3.21 (m, 4H), 1.51 (s, 9H). Combustion
20 Analysis: Found: C, 63.16%; H, 5.56%; N, 9.45%. Calculated for 0.4 mole
21 added water: C, 62.8%; H, 5.7%; N, 9.16%.

22

23 *2-(4-Nitrophenyl)ethyl pyrrolo[4,5-*e*]indoline-7-carboxylate (15).*

24 Two grams (4.43 mmol) of **14** are weighed into a round bottomed
25 flask. Then, in a fume hood, 25 mL (325 mmol) of trifluoroacetic acid is
26 added, and the flask is capped and stirred. The solid dissolves in about a
27 minute. The mixture is stirred for 1 hour, at which time deprotection will be
28 done (HPLC can be used as a check). The acid is evaporated on a rotary
29 evaporator (use a trap) and the product is dissolved in 100 mL of methylene
30 chloride. This is extracted twice with 100 mL of half to 2/3 saturated sodium

1 bicarbonate solution. The aqueous layers are back-extracted once with ~50
2 mL of methylene chloride and this is combined with the rest. The organic
3 layer is dried over sodium sulfate twice and evaporated to give a brown solid.
4 If desired, this material can be recrystallized by diluting a very concentrated
5 solution in methylene chloride with methanol and cooling. Yields
6 approaching 100% are usually obtained. mp 192-194°C. ¹H NMR (DMSO-
7 *d*₆) δ 11.51 (s, 1H), 8.18 (d, J=8.5 Hz, 2H), 7.63 (d, J=8.5 Hz, 2H), 7.11 (d,
8 J=8.5 Hz, 1H), 6.80 (s, 1H), 6.70 (d, J=8.5 Hz), 5.03 (br s, 1H), 4.54 (t, J=6.4 Hz,
9 2H), 3.46 (t, J=8.6 Hz, 2H), 3.19 (m, 2H), 3.04 (t, J=8.6 Hz, 2H). Combustion
10 Analysis: Calculated for C₁₉H₁₇N₃O₄: C, 64.94%; H, 4.88%; N, 11.96%.
11 Found: C, 65.50%; H, 4.70%; N, 11.64%

12

13 2-(4-Nitrophenyl)ethyl 3-({3-[(*tert*-butyl)oxycarbonyl]pyrrolo[4,5-*e*]indolin-
14 7-yl}carbonyl)pyrrolo[4,5-*e*]indoline-7-carboxylate (16).

15 3.09 grams (8.8 mmol) of **15** is mixed with 2.66 grams (8.8 mmol) of
16 **13** (Boger, D.L., Coleman, R.S., Invergo, B.J. (1987) *J. Org. Chem.* 52,
17 1521.), and 46 mL of DMF is added. Then 3.85 grams (8.77 mmol) of 1-[3-
18 (dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride are added. The
19 mixture is stirred for about three hours. The mixture is initially
20 homogeneous, but as the stirring proceeds, a precipitate of the product forms.
21 The solvent DMF is evaporated under a high vacuum, and about 100 mL of
22 methanol is added. The mixture is swirled and filtered in a sintered glass
23 funnel, then thoroughly washed with 3 X 50 mL portions of methanol. Then
24 it is dried in vacuo. Yields usually approach 100 percent. mp: 132E-134°C.
25 ¹H NMR (DMSO-*d*₆) *: 11.93 (s, NH, 1H), 11.62 (s, NH, 1H), 8.30 (br s,
26 aromatic proton, 1H), 8.27 (br s, aromatic proton, 1H), 8.19 (d, aromatic
27 protons, J=8.3 Hz, 2H), 7.65 (d, aromatic protons, J=8.3 Hz, 2H), 7.34 (d, J=9
28 Hz, aromatic proton, 1H), 7.29 (d, J=9 Hz, aromatic proton, 1H), 7.07 (s,
29 aromatic proton, 1H), 6.98 (s, aromatic proton, 1H), 4.60 (m, aliphatic
30 protons, 4H), 4.02 (t, J=8.5 Hz, aliphatic protons, 2H), 3.40 (t, J=8 Hz,

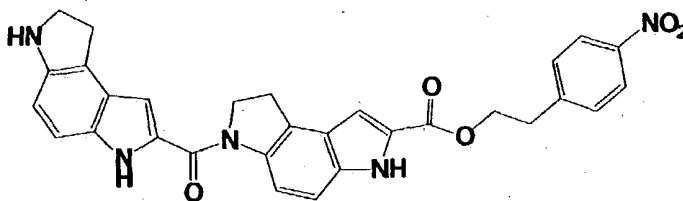
1 aliphatic protons, 2H), 3.24 (m, aliphatic protons, 4H), 1.52 (s, 3xCH₃, 9H).

2 Combustion Analysis: Calculated: C, 66.13%; H, 5.23%; N, 11.02%.

3 Found: C, 65.94%; H, 5.19%; N, 11.07%.

4

5 2-(4-Nitrophenyl)ethyl 3-(pyrrolo[4,5-*e*]indolin-7-ylcarbonyl)pyrrolo[4,5-
6 *e*]indoline-7-carboxylate (17).



7

17

8 5 grams of 16 are placed in a flask. 100 mL of trifluoroacetic acid is
9 added, and the mixture is stirred. After an hour, the acid is evaporated on a
10 rotary evaporator and 100 mL saturated sodium bicarbonate solution and 100
11 mL of water are added. The mixture is agitated or sonicated for ~1/2 hours,
12 then filtered and washed with water and then methanol, and dried in vacuo.
13 The material may be recrystallized. It is dissolved in a minimum amount of
14 warm DMF, and then approximately a threefold portion of methanol is added
15 and the solution is sonicated a few minutes. A cream to brown material
16 crystallizes out. This is washed with methanol, and dried in vacuo. The yield
17 approaches theoretical values. ¹H NMR (DMSO-*d*₆) δ 11.96 (s, NH, 1H),
18 11.71 (s, NH, 1H), 8.30 (br s, aromatic proton, 1H), 8.27 (br s, aromatic
19 proton, 1H), 8.19 (d, aromatic protons, J=8.5 Hz, 2H), 7.66 (d, aromatic
20 protons, J=8.3 Hz, 2H), 7.34 (m, aromatic protons, 2H), 7.08 (s, aromatic
21 proton, 1H), 7.03 (s, aromatic proton, 1H), 4.60 (m, aliphatic protons, 4H),
22 3.68 (t, J=8 Hz, aliphatic protons, 2H), 3.40 (t, J=8 Hz, aliphatic protons, 2H),
23 3.24 (m, aliphatic protons, 4H). Combustion Analysis: Found: C, 63.55 %;
24 H, 4.42 %; N, 11.95 %. Calculated, for ½ mole sodium bicarbonate
25 contaminant: C, 63.43 %; H, 4.45 %; N, 12.13 %.

26

Example 4

2,3,4,5,6-pentafluorophenyl 3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indoline-7-carboxylate (24) (Reaction Scheme 5)

Ethyl 4-[(3-hydroxypropyl)phenylamino]butanoate (19)

A mixture of 3-(phenylamino)propan-1-ol (Huang, Yande; Arif, Atta M.; Bentrude, Weseley G.; *J.Org.Chem.*; 58(23) 1993; 6235-6246) (65.6 g, 0.43 mol), ethyl 4-bromobutyrate (104.5 g, 0.54 mol) and 100 mL of ethyldiisopropylamine is stirred at 100°C for 1 h. The reaction is cooled to room temperature and partitioned between water 400 mL and ethyl acetate (500 mL). The organic layer is washed with saturated NaHCO₃, brine and dried over Na₂SO₄. The oil obtained after concentration is chromatographed on silica eluting with 10% EtOH/CHCl₃. Concentration of the appropriate fractions affords 115 g (100%) of the desired product as a colorless, viscous oil. ¹H NMR (CDCl₃) δ 7.23 (m, 2H), 6.72 (m, 3H), 4.14 (q, J=7 Hz, 2H), 3.72 (t, J=6Hz, 2H), 3.43 (t, 7 Hz, 2H), 3.34 (t, 7 Hz, 2H), 2.35 (t, 7 Hz), 1.88 (m, 4H), 1.26 (t, 7 Hz, 3H).

Ethyl 4-({4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}(3-hydroxypropyl)amino)butanoate (20)

2-Chloro-4-nitroaniline 2.5 g (10 mmol) is placed into a 125 mL flask and 6 mL of water is added. Agitation and sonication partially dissolves the yellow chloronitroaniline. Then the stirred solution is cooled with ice in a fume hood and 15.8 mL of concentrated (~12 M) HCl is added. Most of the yellow material dissolves at this point. The flask is fitted with a dropping funnel, and a solution of 1.51 g (21.9 mmol) sodium nitrite in 3-4 mL of water is added to the dropping funnel and slowly added to the solution in the flask with stirring, over about 20 minutes. When this is complete, 0.6 g (~21

1 mmol) of urea is added followed by 2.73 g of ethyl 4-[(3-
2 hydroxypropyl)phenylamino]butanoate as a solution in 8.2 mL acetic acid.
3 After a minute 20 g of sodium acetate in ~50 mL of water is added. The
4 mixture is allowed to stir for an hour at room temperature. Most of the
5 product is separated as an emulsion. The mixture is partitioned between ethyl
6 acetate and water. The organic layer is washed with NaHCO₃ (3x 50 ml),
7 brine and dried over anhydrous sodium sulfate. Then the organic solvents are
8 evaporated to a syrup. The crude product is chromatographed on silica gel
9 (1.5 x 20 inches) eluting with 50% ethyl acetate/hexane. The appropriate
10 fractions are collected, combined, evaporated (30-40 degrees), and dried in a
11 vacuum. The product is a dark oil. The yield is approximately 68-70%. ¹H
12 NMR (DMSO-*d*₆) δ 8.42 (d, J=2.5 Hz, aromatic proton, 1H), 8.24 (dd, J₁=9
13 Hz, J₂=2.5 Hz, aromatic proton, 1H), 7.86 (d, J=9Hz, 2H), 7.77 (d, J=9 Hz,
14 1H), 6.92 (d, J=9 Hz, aromatic protons, 2H), 4.67 (t, J=6 Hz, OH, 1H), 4.07
15 (q, J=7 Hz, CH₂O, 2H), 3.5 (m, aliphatic protons, 6H), 2.40 (t, J=7 Hz,
16 aliphatic protons, 2H), 1.84 (m, aliphatic protons, 2H), 1.72 (m, aliphatic
17 protons, 2H), 1.18 (t, J=7 Hz, CH₃, 3H).

18

19 4-({4-[(2-Chloro-4-nitrophenyl)diazenyl]phenyl})(3-
20 hydroxypropyl)amino)butanoic acid.

21 To a stirred solution of **20** (4.48 g, 10 mmole) in 40 mL of THF added
22 40 mL of ethanol followed by a solution of KOH (0.84 g, 15 mmol) in 20 mL
23 of water and 20 mL of ethanol. The mixture is stirred overnight and
24 concentrated. The residue suspended in 125 mL of water, treated with 2.6 mL
25 (~ 3 eqv.) of acetic acid, and cooled to 4°C. The resulting solid is filtered off,
26 washed with water, and dried. Yield is quantitative. ¹H NMR (DMSO-*d*₆) δ
27 8.42 (d, J=2.5 Hz, aromatic proton, 1H), 8.23 (dd, J₁=9 Hz, J₂=2.5 Hz,
28 aromatic proton, 1H), 7.82 (d, J=9Hz, 2H), 7.90 (d, J=9Hz, 1H), 7.03 (d, J=9
29 Hz, aromatic protons, 2H), 4.8 (br s, OH, 1H), 3.5 (m, aliphatic protons, 6H),
30 1.86 (t, J=6 Hz, aliphatic protons, 2H), 1.72 (m, aliphatic protons, 4H).

1
2 4-({3-[Bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-
3 nitrophenyl)diazenyl]phenyl}amino)butanoic acid (21)
4 4.21 g (10 mmol) of the acid from the previous step is placed into a
5 250 mL round bottom flask. Dry pyridine (50-100 ml) is added and
6 evaporated (30-40 degrees) with a rotary evaporator. The process is repeated
7 once or twice to remove all traces of water. Dry pyridine (80 mL) is added to
8 the contents of the flask. Then 4.07 g (12 mmol) of dimethoxytrityl chloride
9 is added. After being stirred for 1 h pyridine is evaporated and the resulting
10 syrup is dissolved in a few milliliters of 18:1:1 methylene chloride/methanol/
11 triethylamine. A silica gel column (~1.5" x 20") is prepared with an eluent of
12 18:1:1 methylene chloride/methanol/ triethylamine and the product is run
13 through the column, collecting and combining the appropriate fractions. After
14 the solvents are removed by evaporation the resulting amorphous solid
15 contains some triethylammonium salts in addition to the desired product. The
16 impurity does not interfere with the next step and the product is used without
17 additional purification.

18
19 2,3,4,5,6-Pentafluorophenyl 4-({3-[bis(4-methoxyphenyl)phenylmethoxy]-
20 propyl}{4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoate (22).

21 To the flask containing 21 (10 mmol) is added 7 mL of triethylamine
22 followed by 100 mL of methylene chloride, 2.05 mL of pentafluorophenyl
23 trifluoroacetate (PFP-TFA) is then added. The solution is stirred for half an
24 hour. At the end of this time, the reaction is usually complete. (TLC: 2:1
25 hexane/ethyl acetate). The solvent is removed on the rotary evaporator to
26 give a syrup which is chromatographed on silica eluting with 1:3 ethyl
27 acetate/hexane. Appropriate fractions are collected, combined, evaporated
28 and dried under vacuum. The yield is 41%. ¹H NMR (DMSO-*d*₆) δ 8.43 (d,
29 J=2.5 Hz, aromatic proton, 1H), 8.24 (dd, J₁=9 Hz, J₂=2.5 Hz, aromatic
30 proton, 1H), 7.83 (d, J=9 Hz, aromatic proton, 1H), 7.78 (d, J=9 Hz, aromatic

1 proton, 1H), 7.42-7.15 (m, aromatic protons, 10H), 7.07 (m, aromatic protons,
2 2H), 7.00-6.80 (m, aromatic protons, 4H), 3.72 (s, 2xCH₃, 6H), 3.56 (m,
3 aliphatic protons, 2H), 3.48 (t, J=6.3 Hz, aliphatic protons, 2H), 3.08 (t, J=5
4 Hz, aliphatic protons, 2H), 2.89 (t, J=7 Hz, aliphatic protons, 2H), 1.95 (m,
5 aliphatic protons, 2H), 1.86 (m, aliphatic protons, 2H).

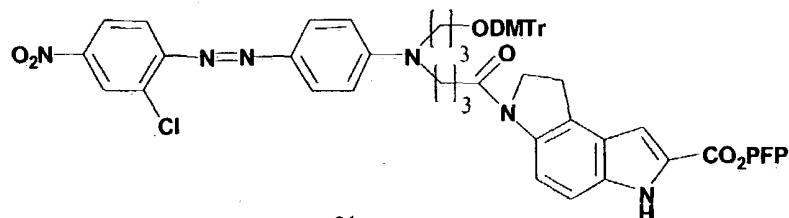
6

7 *Methyl 3-[4-({3-[bis(4-methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-*
8 *4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indoline-7-*
9 *carboxylate (23).*

10 To a solution of 22 (3.0 g, 3.37 mmol) in 15 mL anhydrous DMF is
11 added triethylamine (0.75 mL) followed by methyl pyrrolo[4,5-e]indoline-7-
12 carboxylate (Boger, D.L., Coleman, R.S., Invergo, B.J. (1987) *J. Org.*
13 *Chem.* 52, 1521.) (0.8 g, 3.7 mmol). The resultant solution is stored at room
14 temperature for 20 h. The reaction is analyzed by HPLC to confirm its
15 completeness. DMF is removed on a rotary evaporator equipped with an oil
16 pump. The residue, dark syrup is suspended in 50% ethylacetate/hexanes (~25
17 mL). The mixture is sonicated to initiate the crystallization. The crystals are
18 stirred for 15 min, collected by filtration on a sintered glass funnel, washed
19 with methanol (2x30 mL) and dried under vacuum. The yield of the desired
20 product is 2.7 g (87%) as a deep-purple solid. ¹H NMR (DMSO-*d*₆) δ 11.93
21 (d, J=1.7 Hz, indole NH, 1H), 8.43 (d, J=2.5 Hz, aromatic proton, 1H), 8.3-8.2
22 (m, aromatic protons, 2H), 7.85-7.75 (m, aromatic protons, 3H), 7.45-7.18 (m,
23 aromatic protons, 10 H), 7.05 (d, J=1.8 Hz, aromatic proton, 1H), 6.97 (d, J=9
24 Hz, aromatic protons, 2H), 6.87 (d, J=9 Hz, aromatic protons, 4H), 4.12 (t,
25 J=8 Hz, aliphatic protons, 2H), 3.87 (s, ester CH₃, 3H), 3.71 (s, CH₃, 6H),
26 3.60 (br t, aliphatic protons, 2H), 3.45 (br t, aliphatic protons, 2H), 3.29 (br t,
27 aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (br t,
28 obscured by DMSO signal, aliphatic protons, 2H), 1.88 (br m, aliphatic
29 protons, 4H).

30

1 2,3,4,5,6-pentafluorophenyl 3-[4-((3-[bis(4-methoxyphenyl)phenylmethoxy]-
 2 propyl){4-[(2-chloro-4-nitrophenyl)diazenyl]phenyl}amino)butanoyl]-
 3 pyrrolo[4,5-e]indoline-7-carboxylate (24)



4 24

5 1. Hydrolysis of the methyl ester

6 To a solution of **23** (2.67 g, 2.9 mmol) in 25 mL THF are added
 7 methanol (25 mL) and 5% LiOH, monohydrate in H₂O (10 mL). The
 8 resultant suspension is stirred at 50°C (bath temperature) for 90 min. by which
 9 time a clear solution is obtained. TLC analysis shows no starting material.
 10 Solvent is removed under vacuum and the product is partitioned between
 11 CH₂Cl₂ and cold 10% citric acid. The organic phase is neutralized with
 12 triethylamine, dried over Na₂SO₄ and concentrated. The resultant product
 13 (amorphous solid) is dried in high vacuum for at least 3 h and used in the next
 14 step without additional purification.

15 2. PFP ester preparation

16 The product obtained in the previous step is dissolved in 100 mL
 17 anhydrous DMF. Triethylamine (2 mL) is added followed by PFP-TFA (2
 18 mL, 4.4 mmol). The reaction is stirred for 30 min and analyzed by HPLC. No
 19 starting material, free acid should be observed. DMF is evaporated and the
 20 residue, deep purple syrup is suspended in 100 mL MeOH. After stirring for
 21 30 min, a dark precipitate is formed which is collected by filtration on a
 22 sintered glass funnel, washed with methanol (3x20 mL) and dried under
 23 vacuum (15 - 30 h). This procedure yields 2.7 g (94%) of the desired product
 24 as a purple solid. ¹H NMR (DMSO-*d*₆) δ 12.45 (d, J=1.8 Hz, indole NH,
 25 1H), 8.43 (d, J=2.5 Hz, aromatic proton, 1H), 8.38 (d, J=9 Hz, aromatic
 26 proton, 1H), 8.24 (dd, J₁=9 Hz, J₂=2.5 Hz, aromatic proton, 1H), 7.85-7.75
 27 (m, aromatic protons, 3H), 7.52-7.18 (m, aromatic protons, 11 H), 6.97 (d,

1 J=9 Hz, aromatic protons, 2H), 6.88 (d, J=9 Hz, aromatic protons, 4H), 4.16
 2 (t, J=8.5 Hz, aliphatic protons, 2H), 3.71 (s, CH₃, 6H), 3.61 (br t, aliphatic
 3 protons, 2H), 3.47 (br t, aliphatic protons, 2H), 3.32 (br t, aliphatic protons,
 4 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H), 2.5 (br t, obscured by DMSO
 5 signal, aliphatic protons, 2H), 1.88 (br m, aliphatic protons, 4H).

6 7 Example 5

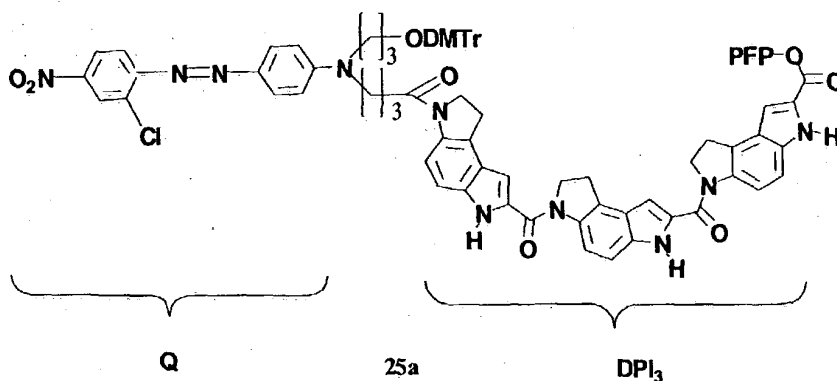
8 2,3,4,5,6-Pentafluorophenyl 3-{{3-{{3-{{4-{{3-{{bis(4-methoxyphenyl)-
 9 phenylmethoxy}propyl}}4-{{(2-chloro-4-nitrophenyl)diazenyl}phenyl}-
 10 amino)butanoyl}pyrrolo[4,5-e]indolin-7-yl}carbonyl}pyrrolo[4,5-
 11 e]indolin-7-yl}carbonyl}pyrrolo[4,5-e]indoline-7-carboxylate (25a). where
 12 R₁= 2-Cl and t=v=3 (Reaction Scheme 6)

13
 14 2-(4-Nitrophenyl)ethyl 3-{{3-{{3-{{4-{{3-{{bis(4-
 15 methoxyphenyl)phenylmethoxy}propyl}}4-{{(2-chloro-4-
 16 nitrophenyl)diazenyl}phenyl}amino)butanoyl}pyrrolo[4,5-e]indolin-7-
 17 yl}carbonyl}pyrrolo[4,5-e]indolin-7-yl}carbonyl}pyrrolo[4,5-e]indoline-7-
 18 carboxylate (25).

19 Into a 100 mL round bottom flask is weighed out 1.31 g (1.22 mmol)
 20 of **24**. This is dissolved in 25 mL of dimethylformamide. Then 0.81 mL of
 21 triethylamine is added, and finally 0.623 g (1.162 mmol) of **17**. The reaction
 22 mixture is left overnight, then the solution is concentrated to ~10 mL and the
 23 resultant precipitate is filtered off, using a sintered glass filter funnel. The
 24 solid is washed with generous volumes of methanol (stirring the sludge in the
 25 filter with the methanol before applying the vacuum) several times and ether.
 26 When the effluent is clear and essentially colorless, the deep violet precipitate
 27 is dried in vacuo to afford 1.5 g (90%) of the desired product. ¹H NMR
 28 (DMSO-*d*₆) δ 11.96 (s, indole NH, 1H), 11.76 (s, indole NH, 1H), 11.69 (s,
 29 indole NH, 1H), 8.43 (d, J=2.4 Hz, aromatic proton, 1H), 8.35-8.20 (m,
 30 aromatic protons, 4H), 8.19 (d, J=9 Hz, aromatic protons, 2H), 7.85-7.75 (m,

1 aromatic protons, 3H), 7.66 (d, J=9Hz, aromatic protons, 2H), 7.45-7.18 (m,
 2 aromatic protons, 12H), 7.10 (s, aromatic proton, 1H), 7.01 (s, aromatic
 3 proton, 1H), 6.99 (m, aromatic protons, 3H), 6.88 (d, J=9 Hz, aromatic
 4 protons, 4H), 4.61 (m, aliphatic protons, 6H), 4.14 (t, J=8.5 Hz, aliphatic
 5 protons, 2H), 3.71 (s, 2xCH₃O, 6H), 3.59 (m, aliphatic protons, 2H), 3.43 (m,
 6 aliphatic protons, 6H), 3.34 (m, obscured by water signal, aliphatic protons,
 7 2H), 3.22 (m, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic protons, 2H),
 8 2.5 (t, obscured by DMSO signal, COCH₂-, 2H), 1.89 (br m, aliphatic protons,
 9 4H). Analysis: Calculated: C, 68.27 %; H, 4.95 %; N, 10.81%. Found: C,
 10 68.08 %; H, 4.98 %; N, 10.63 %.

11
 12 2,3,4,5,6-Pentafluorophenyl 3-{{[3-({[3-[4-({[3-[bis(4-
 13 methoxyphenyl)phenylmethoxy]propyl}{4-[(2-chloro-4-
 14 nitrophenyl)diazenyl]phenyl}amino)butanoyl]pyrrolo[4,5-e]indolin-7-
 15 yl}carbonyl]pyrrolo[4,5-e]indolin-7-yl]carbonyl]pyrrolo[4,5-e]indoline-7-
 16 carboxylate (25a).



17
 18 Into a flask is placed 1.0 g (0.73 mmol) of the product from the
 19 previous step, 40 mL of THF, and 2.46 g of DBU. The mixture is stirred at
 20 50 degrees for 4 hours, then removed from the heat and evaporated to 15 to 20
 21 mL. About 40 mL of methanol is added to the product and the mixture is
 22 agitated and sonicated. Then the precipitate is filtered off with a sintered
 23 glass funnel and washed with 40-60 mL of additional methanol, followed by a
 24 similar amount of ethyl ether, each time stirring the material in the filter prior

1 to applying the vacuum so that the effluent soon becomes clear. The product
2 is dried in vacuo for an hour or two before it is used in the next step. The
3 material is dissolved in 20 mL of DMF in a 100 mL flask and stirred to
4 dissolve. Then 0.6 mL (4.3 mmol) of triethylamine is added, followed by 0.6
5 mL of PFP-TFA. The reaction mixture is stirred under argon overnight, and
6 then evaporated to a gum and a ~10 mL of DMF is added, followed by ~80
7 mL of methanol. This mixture is swirled and sonicated, and then the product,
8 which precipitates out, is filtered off and dried in vacuo. Yield is 85-90%. ¹H
9 NMR (DMSO-*d*₆) δ 12.01 (s, indole NH, 1H), 11.76 (s, indole NH, 1H),
10 11.69 (s, indole NH, 1H), 8.43 (d, J=2.4 Hz, aromatic proton, 1H), 8.40 (br s,
11 aromatic proton, 1H), 8.35-8.20 (m, aromatic protons, 3H), 7.85-7.75 (m,
12 aromatic protons, 3H), 7.59 (d, J=1.2 Hz, aromatic proton, 1H), 7.45-7.18 (m,
13 aromatic protons, 12H), 7.13 (s, aromatic proton, 1H), 6.99 (m, aromatic
14 protons, 3H), 6.88 (d, J=9 Hz, aromatic protons, 4H), 4.66 (m, aliphatic
15 protons, 4H), 4.14 (t, J=8.5 Hz, aliphatic protons, 2H), 3.71 (s, 2xCH₃O, 6H),
16 3.59 (m, aliphatic protons, 2H), 3.43 (m, aliphatic protons, 6H), 3.34 (m,
17 obscured by water signal, aliphatic protons, 2H), 3.08 (t, J=5 Hz, aliphatic
18 protons, 2H), 2.5 (t, obscured by DMSO signal, COCH₂-, 2H), 1.89 (br m,
19 aliphatic protons, 4H). Analysis: Found: C, 63.58 %; H, 4.13 %; N, 9.53 %.
20 Calculated, for 2.3 moles of water: C, 63.97 %; H, 4.21 %; N, 9.44 %.

21

22

Example 6

23

DMTrO-Red 13-amide-CDPI₃-CPG (29) (Reaction Scheme 7)

24

3-[(4-Methoxyphenyl)diphenylamino]propan-1-ol (26).

25

26

27

28

29

30

4 g (53 mmol) of 3-aminopropanol was dissolved by stirring in 50 mL
of methylene chloride in an oven dried 250 mL round bottom flask. This
solution was stoppered and set aside. 7.7 g (24.9 mmol) of
monomethoxytrityl chloride (MMT-Cl, Aldrich reagent grade) was dissolved
in another 50 ml of methylene chloride. An oven dried dropping funnel was
fitted to the flask and the MMT-Cl solution was added to the funnel. The

1 MMT-Cl solution was then added to the solution in the flask over ~10 min
2 (some heat develops). After an hour the reaction was analyzed by TLC (1:1
3 v/v hexanes/ethyl acetate, R_f 0.4) and found to be complete. Visualization of
4 TLC spots by ninhydrin spray / heat showed a trace of (faster moving) bis-
5 MMT side product. The reaction mixture was added to 200 mL of water
6 standing over 200 mL of methylene chloride in a separatory funnel. The
7 mixture was shaken and separated into layers; the aqueous layer was
8 discarded and the organic layer was washed with an additional 200 mL of
9 water. The organic layer was dried over 10-20 g of sodium sulfate and
10 evaporated to give ~ 7 g of the tritylated amine as a pale yellow syrup. This
11 compound did not require further purification and was dried overnight. After
12 several days the syrup solidified. The product was recrystallized from ether-
13 hexanes to give 4.6 g (53% yield) of **26** as a white solid (mp = 89.5 - 90.5
14 EC). Anal. calcd for $C_{23}H_{25}NO_2$: C, 79.51; H, 7.25; N, 4.03. Found: C,
15 79.48; H, 7.18; N, 3.98.

16

17 *2-[(3-[(4-Methoxyphenyl)diphenylamino]propyl)oxycarbonyl)methoxy]-*
18 *acetic acid, triethylammonium salt (27).*

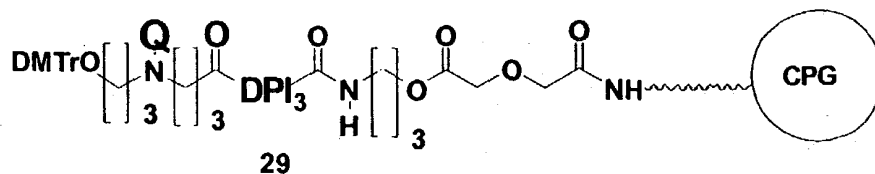
19 2.72g (7.83 mmol) of the alcohol (**26**) was dissolved in 20 mL of
20 methylene chloride with 1.3 mL (9.4 mmol) of triethylamine and 1.1 g (9.5
21 mmol) of glycolic anhydride. The mixture was stirred for 2 h (became
22 homogeneous). TLC showed clean reaction (R_f = 0.35 in 9:1 / methylene
23 chloride:methanol). The solvents were removed by evaporation and the
24 residue was chromatographed on a 1.5 x 18 inch silica gel column packed
25 with 93% methylene chloride, 5% methanol, and 2% triethylamine. The
26 fractions containing product were combined and solvent was removed by
27 evaporation. Co-evaporation with dry DMF ensured removal of traces of
28 water and of residual volatile solvents. Yield of the colorless syrup (**27**) was
29 assumed to be 100%. The syrup was dissolved in dry DMF to give a final
30 volume of 23.4 mL (~ 0.33 M solution).

Synthesis of N-MMT diglycolate CPG (28).

10 g of LCAA-CPG was combined with 5 mL of a 0.33 M solution of **27** in DMF (1.66 mmol) in a 100 mL round bottom flask. A solution of 2.5 mL of diisopropylethylamine, 0.11 g (0.8 mmol) of HOBT and 0.63 g (1.66 mmol) of HBTU was prepared and added to the CPG. The mixture was stoppered and swirled for 16 h on an orbital shaker (150 rpm). The CPG was filtered on a medium porosity sintered glass funnel and washed with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). Unreacted amino groups were acetylated by treating the CPG with 40 mL of dry pyridine and 5 mL of acetic anhydride. After swirling for 1.5 h, the CPG was filtered and washed with 2x 100 mL of DMF, 2x 100 mL of acetonitrile, and 2x 100 mL of ether. Traces of ether were removed in vacuo (oil pump). The CPG was analyzed for MMT loading by treating 3-5 mg of CPG in 25 mL of 1:1 / 70% perchloric acid:methanol. The absorbance of the released MMT cation was recorded at 472 nm and loading level was calculated to be 95.7 :mol / g of CPG using the equation:

$$\text{MMT loading (:mol / g)} = A_{472} \times \text{volume (in mL)} \times 14.3 \div \text{wt of CPG (mg)}$$

Synthesis of CPG 29.



4 g of N-MMT diglycolate CPG (**28**) was weighed into a medium porosity sintered glass funnel. The CPG was detritylated by treating with 25 mL of 3% TCA / DCM. After stirring briefly with a spatula, the mixture reacted for 5 min before filtering (turned yellow). The process was repeated 4 times until the filtrate was colorless. The CPG was washed with 4x 40 mL of

1 methylene chloride. The filtrate was discarded to organic waste, and the CPG
2 was neutralized by treatment with 40 mL of 20% triethylamine in acetonitrile.
3 After briefly stirring with a spatula, the mixture was filtered and washed with
4 2x 40 mL of acetonitrile, and 2x 40 mL of ether. Traces of ether were
5 removed in vacuo (oil pump). The de-tritylated CPG was used immediately
6 for the following immobilization reaction.

7 0.259 g (180 :mol) of **25a** was shaken with 12 mL of dry DMSO in a
8 15 mL polypropylene tube. After 15 min, the dark purple solution was added
9 to 4 g of detritylated diglycolate CPG (in a 50 mL round bottom flask). This
10 corresponds to an offering ratio of 45 :mol PFP ester per gram of CPG. An
11 additional 5 mL of DMSO was added to the polypropylene tube to dissolve
12 residual PFP ester and the solution was added to the CPG. 2 mL of
13 triethylamine was added and the mixture was stoppered and swirled on an
14 orbital mixer for 14 h. The CPG was filtered and washed with 2x 50 mL of
15 DMSO, 2x 50 mL of acetonitrile, and 2x 50 mL of ether. Traces of ether
16 were removed in vacuo (oil pump). Unreacted amino groups were acetylated
17 by treating the CPG with 10 mL of dry pyridine and 3 mL of acetic anhydride.
18 After swirling for 6 h, the CPG was filtered and washed with 2x 50 mL of
19 DMF, 2x 50 mL of acetonitrile, and 2x 50 mL of ether. Traces of ether were
20 removed in vacuo (oil pump). The CPG was analyzed for DMT loading by
21 treating 3-5 mg of CPG in 25 mL of 1:1 / 70% perchloric acid:methanol. The
22 absorbance of the released DMT cation was recorded at 498 nm and loading
23 level was calculated to be 45 :mol / g of CPG using the equation:

24
$$\text{DMT loading (:mol / g)} = A_{498} \times \text{volume (in mL)} \times 14.3 \div \text{wt of CPG (mg)}$$

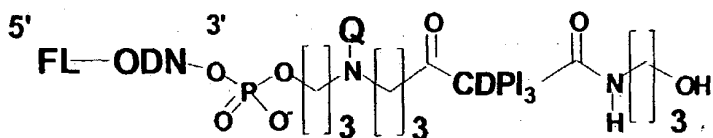
25

26

Example 7

27

Synthesis of FL-ODN-Red 13-amide-CDPI₃ (30).



30

The oligonucleotides were synthesized on the CPG 29 using standard phosphoramidite coupling chemistry except that the standard 0.1 I₂ oxidizing solution was diluted to 0.01-0.015 to avoid iodination of the MGB moiety. FAM and TET were incorporated at the 5' end using the corresponding phosphoramidites available from Glen Research.

Example 8

4-[[N-(6-[[Bis(methylethyl)amino](2-cyanoethoxy)phosphinoxy]hexyl)carbamoyl]methyl]-2-oxo-2H-chromen-7-yl 2,2-dimethylpropanoate (34a).

N-(6-Hydroxyhexyl)-2-(7-hydroxy-2-oxo(2H-chromen-4-yl))acetamide (32a).

(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetic acid methyl ester (**1**) was synthesized according to Baker et al. (*J. Chem. Soc.*; 1950; 170, 173.).

A solution of **31** (2.0 g, 8.5 mmol) and 6-aminohexanol (4.0 g, 34.1 mmol) in 15 mL of DMF was heated at 80°C for 24 h. DMF was evaporated under vacuum to afford the mixture of the product and the excess 6-aminohexanol as a viscous syrup. Chromatography on silica eluting with 10% MeOH/CH₂Cl₂ and evaporation of the pure product fractions afforded a white solid which was washed with ether and dried under vacuum. The yield was 2.05 g (75%).

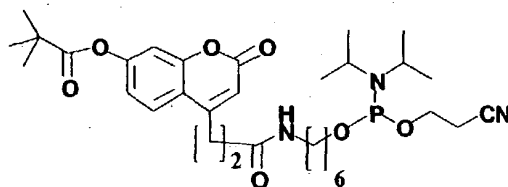
4-[[N-(6-Hydroxyhexyl)carbamoyl]methyl]-2-oxo-2H-chromen-7-yl 2,2-dimethylpropanoate (33a).

To a solution of **32a** (2.0 g, 6.3 mmol) in 20 mL of dry pyridine was added 4,4'-dimethoxytriphenylmethyl chloride (3.0 g, 8.9 mmol). The solution was kept at room temperature for 1 h, TLC analysis (ethyl acetate, R_f 0.7) showed complete reaction (protection of the primary hydroxy group).

1 To this solution was added trimethylacetic anhydride (2.0 mL, 9.9 mmol)
 2 followed by triethylamine (5 mL) and 4-(dimethylamino)pyridine (0.3 g). The
 3 mixture was stirred for 5 h, TLC analysis showed complete protection of the
 4 phenol group ($R_f \sim 0.9$, ethyl acetate). Methanol was added to quench excess
 5 anhydride. Pyridine was removed by evaporation under vacuum and co-
 6 evaporation with xylene. The product obtained was partitioned between ethyl
 7 acetate and 2% NaHCO_3 , the organic phase was concentrated under vacuum
 8 to give the crude DMT protected **33a**.

9 To remove the DMT group the DMT derivative was dissolved in 100
 10 mL of 10% MeOH in CH_2Cl_2 and treated with 0.5 mL of trifluoroacetic acid.
 11 After being stirred for 1 h, the reaction mixture was neutralized with
 12 triethylamine (0.7 mL) and concentrated. The resultant viscous oil was
 13 partitioned between ethyl acetate and water. The organic layer was dried over
 14 Na_2SO_4 and concentrated. The solid obtained was suspended in ether (50 mL)
 15 and stirred for 30 min. The desired product was the insoluble material, and
 16 was collected by filtration, washed with ether and dried. The yield of the title
 17 product **33** was 1.6 g (64%).

18 **4-[[N-(6-[[Bis(methylethyl)amino](2-**
 19 **cyanoethoxy)phosphinoxy]hexyl)carbamoyl]methyl]-2-oxo-2H-chromen-**
 20 **7-yl 2,2-dimethylpropanoate (34).**



34a

21 To a solution of **33a** (0.6 g, 1.5 mmol) in 10 mL of anhydrous CH_2Cl_2
 22 was added triethylamine (0.4 mL) followed by 2-cyanoethyl
 23 diisopropylchlorophosphoramidite (0.35 mL, 1.6 mmol). The solution was
 24 kept at room temperature for 1 h and treated with 0.1 mL of MeOH. The
 25 solvent was evaporated and the residue was partitioned between ethyl acetate
 26

1 and saturated NaHCO₃. The organic phase was washed with saturated NaCl,
2 dried over Na₂SO₄ and concentrated. The crude product was chromatographed
3 on silica eluting with 5% triethylamine in ethyl acetate. Concentration of the
4 pure product fractions and drying under vacuum afforded 0.59 g (65%) of **34**
5 as a colorless, viscous oil.

6

7

Example 9

8 **8-(3-[[bis(methylethyl)amino](2-cyanoethoxy)phosphinoxy]propyl)-7-**
9 **oxophenoxazin-3-yl 2,2-dimethylpropanoate (37a).** (Reaction Scheme 9)
10 *7-Hydroxy-2-(3-hydroxypropyl)phenoxazin-3-one (35a).*

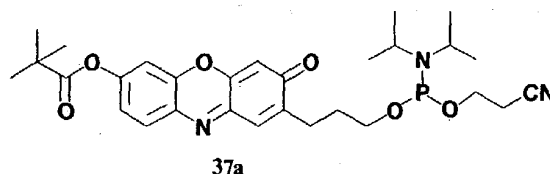
11 A suspension of 4-nitrosorecorcinol (4.5 g, 32.4 mmol), 4-(3-
12 hydroxypropyl)benzene-1,3-diol (Forchiassin, M.; Russo, C., *J. Heterocyc.*
13 *Chem.* 20, 1983, 493-494.) (4.0 g, 23.8 mmol) and MnO₂ (2.5 g, 17.6 mmol)
14 in 50 mL of MeOH was cooled to ~0°C (ice bath). To this suspension was
15 added dropwise 2.5 mL of conc. H₂SO₄ and the reaction was stirred at room
16 temperature for 5 h. The precipitated red resazurin compound was collected
17 by filtration, washed with methanol and dried. The yield was 5.5 g. This
18 product was not homogeneous, it was contaminated with resorufin compound
19 and manganese salts.

20 The crude resazurin compound was suspended in a mixture of 200 mL
21 of water and 50 mL of conc. NH₄OH. Zinc dust (2.0 g) was added and the
22 suspension was stirred for 20 min. The resultant purple mixture was filtered,
23 the filtrate was vigorously stirred on air to oxidize the leuco resorufin, the
24 product of partial over reduction. The reaction was acidified with acetic acid,
25 the brown solid formed was collected by filtration washed with water and
26 dried. The yield was 2.1 g. The material contained ~50% of 2,3,4-trihydro-
27 2H-pyran[3,2-b]phenoxazin-9-one, product of intramolecular cyclization
28 which had been carried over from the first step. The rest of the material was
29 the desired title compound **35**.
30 *8-(3-hydroxypropyl)-7-oxophenoxazin-3-yl 2,2-dimethylpropanoate (36b).*

1 A suspension of **35a** (2.0 g) in 50 mL of pyridine was treated with
2 4,4'-dimethoxytriphenylmethyl chloride (5.0 g, 14.8 mmol) and stirred for 5
3 h. The mixture was filtered to remove some insoluble material and the filtrate
4 was treated with trimethylacetic anhydride (2 mL). The solution was stirred
5 for 15 h and MeOH (2 mL) was added to quench excess anhydride. After
6 being stirred for 3 h, the reaction mixture was concentrated under vacuum.
7 Residual pyridine was removed by co-evaporation with triethylamine and
8 xylene. The resultant crude product **36a** was chromatographed on silica
9 eluting with 50% ethyl acetate/hexane.

10 The DMT derivative was dissolved in 100 mL of 10% MeOH/CH₂Cl₂
11 and treated with 0.5 mL of trifluoroacetic acid. After 1 h, triethylamine (2
12 mL) was added and the solution was concentrated. Chromatography on silica
13 (ethyl acetate) and drying afforded 0.38 g of the desired product as an orange
14 solid.

15 8-(3-{{[bis(methylethyl)amino](2-cyanoethoxy)phosphinoxy}propyl)}-7-
16 oxophenoxazin-3-yl 2,2-dimethylpropanoate (**37a**).



17
18 **36b** (0.38 g, 1.1 mmol) was dissolved in 6 mL of anhydrous CH₂Cl₂.
19 Triethylamine (1.5 mL) was added followed by 2-cyanoethyl
20 diisopropylchlorophosphoramidite (0.29 mL, 1.3 mmol). The solution was
21 kept at room temperature for 30 min, MeOH (0.1 mL) was added and the
22 reaction was concentrated under vacuum. The residue obtained was
23 partitioned between ethyl acetate and NaHCO₃. The organic phase was
24 washed with saturated NaCl, dried over Na₂SO₄ and concentrated to afford
25 the crude amidite. It was dissolved in 2 mL of ether and added dropwise to ~
26 50 mL of hexane. The resultant orange solid was collected by filtration,
27 washed with hexane and dried. The yield was 0.4 g.

28

Example 10

3-[[[(tert-Butyl)(methylethyl)amino][2-(4-{3-butyl-7-[(4-methoxyphenyl)-carbonyl]-2,4,6,8-tetraoxo-1-(phenylcarbonyl)(1,3,5,7,9,10-hexahydro-pyrimidino[5',4'-5,6]pyridino[2,3-d]pyrimidin-10-yl)}phenyl)ethoxy]-phosphinoxy}propanenitrile (PPT) 44 (Reaction Scheme 10)

3-n-Butyl-6-[4-(2-hydroxyethyl)aminophenyl]uracil 40.

A mixture of 6-chloro-3-n-butyluracil (10.4 g, 51.3 mmol), 2-(4-aminophenyl)ethanol (10.0 g, 72.9 mmol) and ethyldiisopropylamine (18 ml, 0.1 mol) was heated with stirring under argon on a 150° oil bath for 1 hr 20 min. The mixture was cooled to room temperature, diluted with 50 ml of water, treated with 10 ml of acetic acid and stirred for crystallization overnight. A precipitated solid was filtered, washed with 2% acetic acid, dried on filter and dissolved in 100 ml of hot 96% ethanol. To the solution 100 ml of hot water was added followed by 1.0 g of charcoal. The mixture was filtered hot and crystallized on ice. Yellow solid was collected by filtration and dried in vacuum to yield 10.7 g of **40**, mp 207-208 °C. ¹H NMR (DMSO-d₆) δ 0.88 (t, 3H, *J* = 7.3 Hz, CH₃), 1.25 (m, 2H, CH₂), 1.46 (m, 2H, CH₂), 2.70 (t, 2H, *J* = 6.8 Hz, CH₂), 3.60 (dd, 2H, *J* = 11.8, 6.8 Hz, CH₂), 3.68 (t, 2H, *J* = 7.3 Hz, CH₂), 4.62 (t, 1H, *J* = 5.3 Hz, OH), 4.73 (d, 1H, *J* = 1.8 Hz, 5-H), 7.10 (d, 2H, *J* = 8.4 Hz, ArH), 7.23 (d, 2H, *J* = 8.4 Hz, ArH), 7.10 (s, 1H, NH), 10.37 (s, 1H, NH).

3-n-Butyl-10-[(2-hydroxyethyl)phenyl]pyrido[2,3-d;6,5-d']dipyrimidine-2,4,6,8-(3H,7H,9H,10H)-tetrone.41

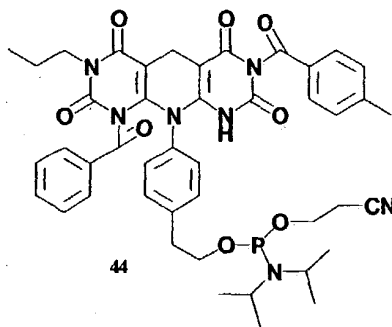
A solution of **40** (6.6 g, 20 mmol) and 5-formyl-2,4,6-trichloropyrimidine (5.85 g, 27.7 mmol) in 80 ml of dry DMF was stirred at RT for 8 hr and slowly diluted with 80 ml of water. The solution produced a solid upon refrigeration for 2 days. The product was isolated by filtration, washed with cold 50% ethanol (50 ml) and 25% ethanol (50 ml) and dried in vacuum to yield 8.16 g (96%) **41** as a colorless solid, mp 205-215 °C

1 (decomp). ^1H NMR (DMSO-d_6) δ 0.88 (t, 3H, $J = 7.2$ Hz, CH_3), 1.27 (m, 2H,
2 CH_2), 1.48 (m, 2H, CH_2), 2.81-2.90 (m, 2H, CH_2), 3.71-3.85 (m, 4H, CH_2),
3 4.50 (br. s, 5H, OH, NH, H_2O), 7.26 (d, 2H, $J = 8.4$ Hz, ArH), 7.44 (d, 2H, J
4 $= 8.4$ Hz, ArH), 8.62 (s, 1H, 5-H).

5
6 *3-n-Butyl-5,10-dihydro-10-[(2-hydroxyethyl)phenyl]pyrido[2,3-d;6,5-*
7 *d']dipyrimidine-2,4,6,8-(1H,3H,7H,9H,10H)-tetrone. 42*

8 To a suspension **41** (7.91 g, 18.7 mmol) in 300 ml of 25% aq. NH_3 was
9 added $\text{Na}_2\text{S}_2\text{O}_4$ (13.8 g, 85%, 67 mmol) and slowly heated to 60° with stirring.
10 The mixture was stirred at 60° for 40 min, diluted with water (100 ml) and
11 stirred for additional 1 hr at the same temperature. A clear solution formed.
12 The solution was partially evaporated to one half of its original volume,
13 cooled with ice and neutralized with 50 ml of acetic acid to pH 5 to form a
14 precipitate. The mixture was kept in refrigerator for complete crystallization,
15 filtered and washed with cold water. The solid was dried in vacuum to yield
16 7.32 g (92%) of **42** as a white solid, mp $182\text{--}210^\circ\text{C}$ (decomp). ^1H NMR
17 (DMSO-d_6) δ 0.86 (t, 3H, $J = 7.3$ Hz, CH_3), 1.23 (m, 2H, CH_2), 1.42 (m, 2H,
18 CH_2), 2.80 (t, 2H, $J = 6.6$ Hz, CH_2), 3.14 (s, 2H, 5- CH_2), 3.68 (m, 4H,
19 ArCH_2CH_2), 4.64 (t, 1H, OH), 7.25 (d, 2H, $J = 8.3$ Hz, ArH), 7.33 (d, 2H, $J =$
20 8.3 Hz, ArH), 7.73 (br. s, 3H, NH).

21
22 **PPT 44**



23
24 Solid **42** (1.2 g, 2.82 mmol) was evaporated with pyridine (10 ml),
25 suspended in pyridine (13 ml), treated with Me_3SiCl (2.2 ml, 17.3 mmol) and

1 stirred under argon at ambient temperature for 30 min. The reaction mixture
2 was cooled with ice and treated slowly with toluoyl chloride (5 ml, 28.8
3 mmol). Stirring was continued at room temperature for 2 hr, and the solvent
4 evaporated. The residue was treated with acetic acid (10 ml) followed by
5 addition of water (10 ml). Precipitated oil was extracted with hexanes (3x50
6 ml), and the residue that was insoluble in hexanes was evaporated with water.
7 The residue was suspended in 96% ethanol (10 ml) and filtered to recover 0.3
8 g of the starting material. The mother liquor was diluted with water to
9 precipitate bis-toluoyl derivative as an oil. The oil was dried in vacuum to
10 give 0.96 g (52%) of **43** as a solid foam. This compound without further
11 purification was converted into phosphoramidite by the following procedure.
12 The solid was evaporated with acetonitrile, dissolved in 25 ml of
13 dichloromethane, treated with diisopropylammonium tetrazolide (0.54 g, 3.13
14 mmol) followed by 2-cyanoethyl tetraisopropylphosphorodiamidite (0.88 g,
15 2.9 mmol). The reaction mixture was stirred under argon for 1 hr, treated with
16 methanol (1 ml), taken into EtOAc (100 ml), washed with sat. NaCl solution
17 and dried over Na₂SO₄. The solution was evaporated, purified by HPLC on
18 silica gel column using a gradient system 0-50% B; CH₂Cl₂-hexanes-NEt₃
19 (15:30:1) (A); EtOAc (B); detected at 320 nm. The main fraction was
20 evaporated giving a colorless foam, 0.79 g (33%) of AG1 phosphoramidite
21 **44**. ¹H NMR (CDCl₃) δ 0.92 (t, 3H, J = 7.3 Hz, CH₃), 1.07-1.42 (m, 14H,
22 4xCH₃ (i-Pr), CH₂ (Bu)), 1.50-1.65 (m, 2H, CH₂ (Bu)), 2.35-2.60 (m, 2H,
23 CH₂CN), 2.40 (s, 3H, CH₃Ar), 2.46 (s, 3H, CH₃Ar), 2.95-3.13 (m, 4H, 2xCH
24 (i-Pr), CH₂ (Bu)), 3.45-3.60 (m, 2H, OCH₂), 3.80-4.02 (m, 4H, ArCH₂CH₂),
25 3.82 (s, 2H, 5-CH₂), 7.15-7.35 (m, 8H, ArH (Tol)), 7.45 (s, 1H, NH), 7.73 (br.
26 s, 3H, NH), 7.95 (d, 2H, J = 8.0 Hz, ArH), 8.05 (d, 2H, J = 8.0 Hz, ArH). ³¹P
27 NMR (CDCl₃) δ (ppm, H₃PO₄) 143.2 (s).

28

29

Example 11

30

N-{3-[4-[(1Z)-1-aza-2-(dimethylamino)prop-1-enyl]-1-(5-{[bis(4-

1 **methoxyphenyl)phenylmethoxy}methyl}-4-{[bis(methylethyl)amino](2-**
2 **cyanoethyl)phosphinoxy}oxolan-2-yl)pyrazolo[5,4-d]pyrimidin-3-**
3 **yl]propyl}[2-({4-[(2-chloro-4-nitrophenyl)diazanyl]phenyl}ethylamino)-**
4 **ethoxy]carboxamide (50) (Reaction Scheme 11)**

6 *4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-trifluoroacetimido-*
7 *propyn-1-yl) pyrazolo[3,4-d]pyrimidine (46; n=1).*

8 To a mixture of **45** (1.96 g, 5.20 mmol), CuI (103 mg, 0.54 mmol) and
9 tetrakis[triphenylphosphine]palladium[0] (317 mg, 0.276 mmol) in 10 ml of
10 anhydrous DMF was added anhydrous triethylamine (1.1 ml) followed by
11 propargyl trifluoroacetimide (1.50g, 9.88 mmol). The reaction mixture was
12 stirred under argon for 4 h. The solvent DMF was removed by evaporation
13 and the residual oil was purified by silica gel chromatography eluting with 7%
14 methanol in ethyl acetate. The product fractions were pooled and evaporated
15 affording a foam: 2.16 g (99%) yield.

17 *4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-*
18 *aminopropyl)pyrazolo[3,4-d]pyrimidine (47; n=1).*

19 To a solution of **46** (2.10 g, 5.25 mmol) in 50 ml of ethanol, containing
20 0.300 mg of 5% palladium on carbon (preactivated with formic acid), was
21 added 1.0 ml of 4 M triethylammonium formate buffer (pH 6.5). The mixture
22 was shaken under 40 psi of hydrogen gas for 18 h. The mixture was filtered
23 through Celite and the filtrate was evaporated affording a solid. 1.8 g (85%)
24 yield.

25 The solid was stirred in 15 ml of concentrated ammonium hydroxide
26 (sealed flask) for 12 h and then evaporated to dryness. The solid (**47**) was
27 evaporated from dry acetonitrile and stored under vacuum: 1.74 g yield.

28
29 *Synthesis of 48 (n=1, q=2, R₅=CH₃CH₂-, R₅, R₅=H, R₁=2-Cl, R₅=4-NO₂).*

30 A solution of **47** (0.90 g, 2.92 mmol) and **7** (1.59 g, 2.92 mmol) was

1 stirred in 5.0 ml of anhydrous dimethylformamide, containing 1.0 ml of
2 triethylamine, at 50 °C for 1.0 h. The solution was evaporated to dryness and
3 the residue was purified by silica gel chromatography eluting with a gradient
4 of 0-20% methanol in ethyl acetate. The product fractions were evaporated
5 affording an amorphous solid: 0.74 g (37%) yield.

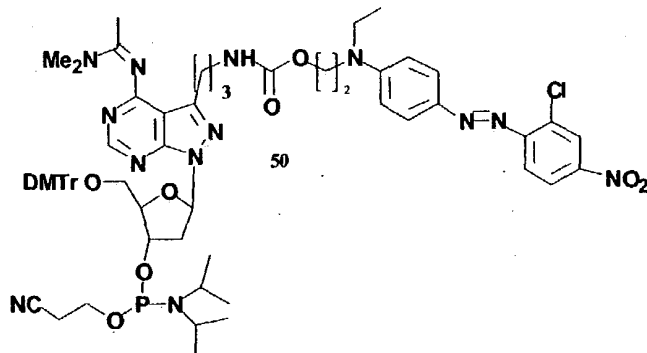
6

7 Synthesis of 49 ($n=1$, $q=2$, $R_5=CH_3CH_2-$, $R_5, R_5=H$, $R_1=2-Cl$, $R_5=4-NO_2$).

8 To a solution of 48 (0.71 g, 1.03 mmol) and N,N-dimethylacetamide
9 dimethylacetal (1.9 ml) in 5.0 ml of dimethylacetamide was added 2.0 ml of
10 triethylamine. The solution was stirred for 18 hrs and then evaporated to
11 dryness affording an oil: 0.75 g (100%) yield.

12

13 Synthesis of 50 ($n=1$, $q=2$, $R_5=CH_3CH_2-$, $R_5, R_5=H$, $R_1=2-Cl$, $R_5=4-NO_2$).



14

15 Dimethoxytrityl chloride (0.42 g) was added to a solution of 49 (0.75
16 g, 1.03 mmol) in 10 ml of dry pyridine. The solution was stirred for 4.0 hr
17 under argon and then poured into 200 ml of 5% sodium bicarbonate solution.
18 The product was extracted with 300 ml of ethyl acetate. The extract was
19 dried over sodium sulfate and evaporated. The residue was purified by silica
20 gel chromatography eluting with 10% methanol in ethyl acetate (1%
21 triethylamine). The product fractions were evaporated affording a foam: 556
22 mg (57%) yield.

23 To a solution of the 5'-dimethoxytrityl derivative (540 mg, 0.567
24 mmol) in 15 ml of anhydrous methylene chloride, containing 0.30 ml of

1 diisopropylethylamine, was added 2-cyanoethyl N,N-
2 diisopropylchlorophosphoramidite (0.25 ml). After stirring for 30 minutes
3 under argon at 25 °C the solution was treated with 1.0 ml of methanol and
4 diluted with 200 ml of ethyl acetate. The solution was washed with 200 ml of
5 5% sodium bicarbonate solution and dried over sodium sulfate and
6 evaporated. The crude product was purified by silica gel chromatography
7 eluting with 5% methanol in ethyl acetate (2% triethylamine). The product
8 fractions were evaporated affording a foam: 453-mg (76%) yield.

9

10 **Example 12**

11 ***Synthesis of fluorogenic oligodeoxynucleotide probes.***

12 The 3'-DPI₃ probes were prepared by automated DNA synthesis from
13 a DPI₃-modified glass support using methods described earlier (Lukhtanov et
14 al. *Biorg. Chem.*, 7: 564-567 (1996)). Oligonucleotide synthesis was
15 performed on an ABI 394 synthesizer according to the protocol supplied by
16 the manufacturer except that 0.015 M (instead of the standard 0.1 M) iodine
17 solution was utilized in the oxidation step to avoid iodination of the CDPI₃
18 moiety. To prevent extension during PCR, probes without 3'-CDPI₃ were
19 prepared with the 3'-hydroxyhexyl phosphate as previously described
20 (Gamper et al. *Biochem.* 36: 14816-14826 (1997)). The quencher
21 phosphoramidites were added to the CPG and standard β-
22 cyanoethylphosphoramidites and reagents (Glen Research, Sterling, VA) were
23 used in oligonucleotide synthesis. 6-Carboxyfluorescein (6-FAM)
24 phosphoramidite (Glen Research) was used to introduce the 5'-reporter dyes.
25 Alternatively TAMRA-dU phosphoramidite (Glen Research), cy3 or cy5
26 phosphoramidite (Glen Research), resorufin phosphoramidite, coumarin
27 phosphoramidite, or AG phosphoramidite was used to introduce the indicated
28 5'-fluorophore. 5'-Hexylamine phosphoramidite (Glen Research) was
29 incorporated into certain ODNs for post-synthetic conjugation of the 3'-
30 quencher dye tetramethylrhodamine (TAMRA). After deprotection, all

1 oligonucleotides were reverse-phase HPLC purified and isolated as the
2 sodium salts by butanol concentration / sodium perchlorate precipitation
3 (Milesi et al. *Methods Enzym.* **313**: 164-173 (1999)).
4

5 **Example 13**

6 **Post-synthetic conjugation of ODNs with TAMRA.**

7 TAMRA NHS ester (Glen Research) was used to acylate the
8 hexylamine linkers in certain ODNs according to the protocol supplied by the
9 manufacturer. The resulting CDPI₃-probes with two conjugated dyes were
10 purified by denaturing gel electrophoresis using 8% polyacrylamide. The
11 desired bands were excised and the gel slices were incubated overnight at
12 37°C in 10 mL of 100 mM Tris-HCl, 10mM triethylammonium chloride, 1
13 mM EDTA (pH 7.8). The products were isolated from the extract by reverse
14 phase HPLC, butanol concentration and sodium perchlorate precipitation.
15 The pellets were dissolved in water and the concentrations were determined
16 spectrophotometrically. A nearest neighbor model (Cantor, et al.
17 *Biopolymers* **9**: 1059 - 1077 (1970) was applied to calculate extinction
18 coefficients (ϵ_{260}) of ODNs. For the conjugates and probes, extinction
19 coefficients were calculated as a sum of ϵ_{260} for the ODN and the incorporated
20 residues of DPI₃ ($68,000 \text{ M}^{-1}, \text{ cm}^{-1}$), 6-FAM ($22,800 \text{ M}^{-1}, \text{ cm}^{-1}$), TAMRA
21 ($34,000 \text{ M}^{-1}, \text{ cm}^{-1}$) and quencher ($11,300 \text{ M}^{-1}, \text{ cm}^{-1}$).
22

23 **Example 14**

24 **Digestion of oligonucleotides by Snake Venom Phosphodiesterase.**

25 Oligonucleotides were digested with snake venom phosphodiesterase
26 (PDE) to study the fluorescence quenching potential of various quenchers.
27 200 nM of oligonucleotide was taken in a buffer containing 40 mM of NaCl,
28 20 mM of Tris (pH 8.9) , 5mM of MgCl₂ and 0.025% of BSA. Initial
29 fluorescence was read on a LS50B fluorimeter (Perkin-Elmer Corporation,
30 Foster City, CA) before the addition of phosphodiesterase (Pharmacia,

1 Piscataway, NJ) 54 units of enzyme was added to the reaction mixture and
2 incubated at 37 °C for 16 hrs. The final fluorescence was then measured
3 using the LS50B. The ratio of final fluorescence to the initial fluorescence
4 represents the signal to noise ratio (S/N) of the quenchers. Independently the
5 kinetics of digestion reactions were monitored using the LS50B to determine
6 the time required for complete digestion of oligonucleotides.

7

8

Example 15

9

5' Nuclease PCR Assay.

10 CDPI₃-conjugated oligonucleotides were conjugated with a
11 fluorophore, FAM at the 5' end and various quenchers were conjugated
12 through a linker at the 3' end by the methods discussed above. 5' nuclease
13 assays were performed with the above oligonucleotides to determine the
14 quenching ability of the various quenchers under investigation. Fluorescent
15 monitoring was performed in an Idaho Technologies LC-24 LightCycler.
16 Each reaction contained PCR buffer (40 mM NaCl, 20 mM Tris HCl, pH 8.9,
17 5 mM MgSO₄, 0.05% bovine serum albumin), 125 mM each dNTP, 0.5 mM
18 each primer, 0.1 mM fluorescent CDPI₃ probe, 0.5 U/10 mL *Taq* polymerase
19 and 0.1 ng/10 mL of synthetic DNA as template. The cycling program was
20 50 cycles (or as indicated) of 2 sec at 95EC, then 30 sec at the extension
21 temperature (55-70E).